ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL CANCER INSTITUTE
Fiscal Year 1980
Part III-A

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service National Institutes of Health

ANNUAL REPORT

OF

PROGRAM ACTIVITIES,

NATIONAL CANCER INSTITUTE.

Fiscal Year 1980

Part III-A

Division of Cancer Cause and Prevention

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ANNUAL REPORT

DIVISION OF CANCER CAUSE AND PREVENTION

NATIONAL CANCER INSTITUTE

October 1, 1979 through September 30, 1980

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ANNUAL REPORT

DIVISION OF CANCER CAUSE AND PREVENTION

Gregory T. O'Conor, M.D., Director

October 1, 1979 through September 30, 1980

OVERVIEW

Two years ago, the Division of Cancer Cause and Prevention was reorganized according to a plan which clearly separated the intramural and extramural programs. Even though the plan was not yet officially approved at the Department level, the operations of these programs have been carried out as distinct entities since that time. In July 1980, the plan was officially approved; a chart reflecting the new organization is given in Figure 1 (p. 4).

The intramural research program consists of two broad areas of investigation, Carcinogenesis and Epidemiology. Within the first area, four laboratories conduct research in biological carcinogenesis and five laboratories conduct investigations in chemical carcinogenesis. In the second area, three branches are responsible for research in environmental cancer using epidemiologic and biometric approaches; these branches also provide support to the entire Institute.

The extramural component, known collectively as the Carcinogenesis Extramural Program, comprises several major activities: Biological Carcinogenesis, Chemical and Physical Carcinogenesis, and Epidemiology, including major aspects of the Smoking and Health and the Diet and Nutrition Programs. The overall budget for research in these areas is given in Table 1 (p. 5) and Figure 2 (p. 6).

This year the Division embarked upon a plan to return all intramural laboratories to government facilities. The Division once had six laboratory units in leased facilities near the Bethesda reservation; only three groups presently remain in rental space. The plan now in operation will help to accomplish other goals: (1) reduce the cost of contracts now needed to support intramural laboratories, thus decreasing the total cost of supporting our intramural programs; (2) make more extensive use of government-owned facilities at the FCRC; and (3) relieve the overcrowded conditions in laboratories now housed in Building 37, NIH. Ultimately, this plan will bring NCI to the NIH or FCRC reservations and provide additional space in Building 37 for the laboratories of other NCI Divisions, as requested by the Director, NCI.

To accomplish this plan the Division proposes to: (1) relocate the Laboratory of Viral Carcinogenesis to FCRC and (2) relocate sections of the Laboratory of Cellular and Molecular Biology to Building 37. The many details of this proposal are not included in this report because they are not yet fully developed; however, they will be reported next year. When realized, this plan will return all remaining off-reservation DCCP laboratories; provide geographic unity of individual laboratories; help integrate biological, chemical and physical carcinogenesis programs; increase use of

FCRC by government scientists; and reduce funds now needed for resource/ support laboratories. We anticipate no additional requirements for personnel and funds to implement this effort.

The past year has seen a continued reduction in overall contract support. This has been realized by gradual phase-out of contract-supported, investigator-initiated research in areas where grants provide adequate coverage; by reducing activities which provide materials and services; and by initiating various cost-recovery mechanisms. By the same token, as support for research contracts has dropped, support for investigator-initiated research grants has continued to increase.

In addition to its intramural and extramural research, the Division has been involved in other activities during the past year which merit attention. These are:

1. Frederick Cancer Research Center (FCRC)

   Research conducted by the contractor began in the early 1970's as a series of unrelated tasks or projects. Today, the carcinogenesis program at the FCRC has gradually evolved into a coordinated effort on the mechanisms of action of viral and chemical carcinogens. As a separate effort, the contractor provides support services and materials to intramural investigators who work in NCI laboratories at the Frederick facility. Government and contractor scientists have combined their efforts to create a center of excellence for cancer research.

2. National Toxicology Program (NTP)

   Last year the NTP was established as a new organizational entity. Because this interagency consortium posed some unusual management problems, the staff was "detailed" to the NTP in September 1979. The Director of this Program, now known as the Bioassay Program, reports directly to the Director, NTP rather than to the Director, DCCP. For the duration of the experiment, DCCP is no longer involved in NTP operations.

3. International Cancer Research Data Bank (ICRDB)

   In accordance with recommendations of the Reorganization Task Force, the Director, NCI approved the transfer of the ICRDB from the Office of International Affairs, OD, to OD, DCCP. This decision reflects the opinion that the ICRDB, which was operating effectively under Dr. O'Conor should be relocated to his Division; the Program Director and his staff were transferred at the beginning of the fiscal year.

4. The DCCP Board of Scientific Counselors

Chartered in 1978, the Board is an advisory body whose members are drawn from the scientific community outside NIH. Chosen for their recognized expertise in chemical carcinogenesis, molecular biology, viral oncology, epidemiology, immunology, pathology, genetics, and cell biology, the members of the Board advise the Division Director on a wide variety of matters which concern the progress of the Division's programs.

One particularly important responsibility of the Board has been the examination of the productivity and performance of staff scientists by site visit review of the intramural laboratories. These visits have been conducted by teams which, as a rule, comprise two members of the Board and four or more investigators from the scientific community outside NIH whose special fields of expertise match those of the scientists in the Laboratory or Branch being reviewed. The site visit reports, which reflect a consensus of the members of the team, are critically examined by the entire Board of Scientific Counselors and, after discussion, recommendations based on the reports are submitted to the Division Director.

The first cycle of site visits to the Division's entire intramural operation was completed this year with the exception of the site visit to the Laboratory of Viral Carcinogenesis, which was cancelled by the Acting Director, NCI, because of the Laboratory's imminent move to FCRC. Many of the Board's recommendations resulting from these site visits have already been implemented, while still others are in progress. It is anticipated that the next cycle of site visits will begin in 2-3 years.

Another important function of the Board is that of concept review, in which the members pass judgment on concepts for grant- or contract-supported activities. The Board completed concept review of the Division's ongoing extramural program this year, and will continue to examine new concepts as new directions in extramural research activities develop.

As a result of the review of the Surveillance, Epidemiology, and End Results (SEER) Program by the Norris Cotton Cancer Center, and the site visit to the Biometry Branch, DCCP, which manages the SEER Program, the Board recommended that a task force be appointed to review the SEER program and determine how it can best be modified so as to more effectively monitor the cancer experience in the United States. Such a task force has been created, in the form of a subcommittee to the Board headed by Mr. Seymour Jablon. The results of its review are expected early next year.

The Division is most grateful to the members of the Board for giving so generously of their time, effort, and expertise in helping to guide the future of the Division's programs. There is every expectation that the Board will continue to play a vital role as the Division's foremost advisory group.

Figure 1

DCCP ORGANIZATIONAL CHART

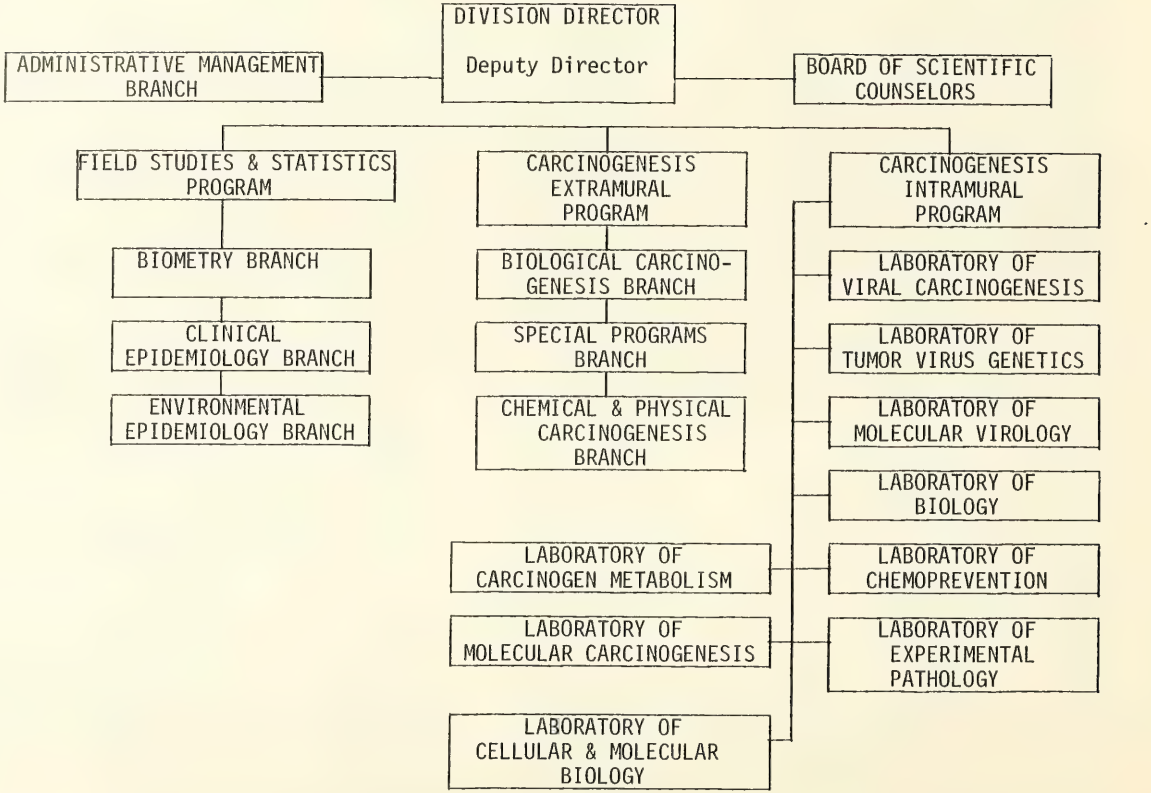


Table 1

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER CAUSE AND PREVENTION

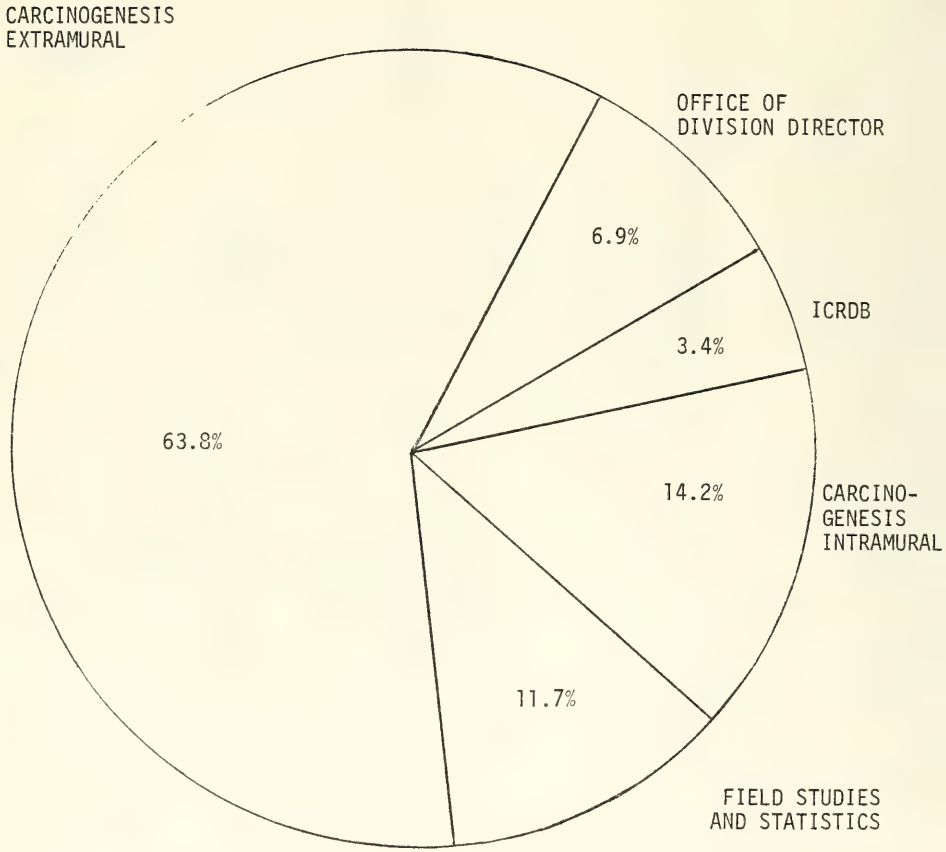
Table of Ratios of Contracts to
Intramural Efforts Based on
Estimated Current Level of Expenditures
(In Thousands)

September 30, 1980

	ODD	Field Studies and Statistics	International Cancer Research Data Bank	Carcinogenesis Intramural	Carcinogenesis Extramural	Total
Total	15,585	26,546	7,799	32,428	57,177	139,535
Contracts	12,052	18,767	7,230	8,459	53,236	99,744
CREG		700			1,312	2,012
Intramural	3,533	7,079	569	23,969	2,629	37,779

Figure 2

NATIONAL CANCER INSTITUTE
Division of Cancer Cause and Prevention
Current Distribution of Funds - Fiscal Year 1980



SCIENTIFIC HIGHLIGHTS

The primary thrust of the Division's research program is predicated on the idea that cancer can be most effectively prevented when its causes and the mechanisms of transformation are understood. The research highlights which follow should be read in this context. Research activities within the Division rest on the understanding that most cancer begins with an alteration in the genetic component of a normal cell and progresses through a series of changes before the clinical appearance of the disease. Although the specific changes are not yet known, it is nevertheless clear that the development of clinically apparent cancer is a stepwise process, and prevention may be possible by interfering with any of the steps in this process. Detailed reports of the research aimed at elucidating the mechanism of carcinogenesis are given later in this report; however, selected highlights of the progress of the Division's entire research program are presented in the following section as a state-of-the-art narrative.

Biological Carcinogenesis

In 1910, Peyton Rous first described the transmissable sarcoma of the common chicken, whose causative agent, later identified as a virus, bears his name. Greeted at first with disbelief, the discovery that viruses can cause cancer gradually gained widespread acceptance as newly isolated agents were found to be linked to malignancy in species ranging from fish and amphibians to non-human primates. The logical assumption that such viruses might also exist for humans reached maturity in 1964, when the NCI began the Special Virus - Leukemia Program, whose objective was the isolation of a human cancer-causing virus and the development of means for the prevention and/or control of human leukemia.

Since that time, unforeseen scientific developments have caused many old ideas to be modified or abandoned entirely. The primary emphasis in biological carcinogenesis has moved from the search for human cancer viruses to the study of viral genes, gene products, and cellular control mechanisms, and tumor viruses are now increasingly regarded as tools for deciphering the molecular pathways by which a normal cell is transformed into a malignant one. The isolation of viral proteins responsible for malignant transformation and the identification of the genetic sequences that code for them have placed at the disposal of the research scientist reproducible means by which the molecular mechanisms of cancer can be scrutinized. Moreover, the discovery that some of the most potent oncogenic viruses derive the information coding for their transforming proteins from normal cells by genetic recombination provides a unique opportunity to isolate and analyze cellular genes involved in tumorigenesis.

Some of the most exciting and significant work in this area has been done with two rat-derived agents, Kirsten sarcoma virus (KiSV) and Harvey sarcoma virus (HaSV), and the Rous sarcoma virus (RSV). A protein, called viral src p21, has been shown to be the protein responsible for cellular transformation by these viruses. Similarly, a protein designated viral src p60 has been

isolated from RSV and shown to be responsible for that agent's transforming potential. These proteins appear to share an important characteristic, in that their genes were originally derived from normal rat or chicken cells by recombination. On the other hand, they have striking differences: viral src p60 uses ATP and phosphorylates tyrosine residues, while viral src p21 binds with high affinity to the guanine nucleotides GTP and GDP and phosphorylates threonine residues. Moreover, it will not use ATP or ADP.

As a GTP-specific kinase, the cancer-inducing viral src p21 protein is a biologically unique molecule. But more importantly, a normal protein which is related to viral src p21 has been found in all normal vertebrate cells, including those of humans. There are, however, qualitative and quantitative differences between the endogenous p21 and the viral src p21, and the detailed biochemistry of each protein is being studied to define the molecular pathway involved in the induction of cancer by viral src p21.

There is also a normal protein in human cells which is related to viral src p60. It is therefore reasonable, since both p60 and p21 are expressed in human cells, to expect that some human malignancies may be found to be caused by each protein, and the comparative biochemistry of these viral transforming proteins now provides investigators two unique and distinct parameters with which to investigate human cancers. For example, phosphotyrosine-containing proteins and GTP-specific protein kinase activity can now be explored for possible use as markers in new tests for the differential diagnosis of human cancers. And in a larger sense, research on p60 and p21 has given a new impetus to studies to develop a rational biochemical basis for the early diagnosis and classification of human tumors, as well as suggesting means for monitoring their clinical course and controlling them.

Research on sarcoma viruses has produced extremely important insights into the mechanisms of biological carcinogenesis. The entire genome of another of these agents, Moloney sarcoma virus (MSV), has been cloned and amplified by recombinant DNA techniques. More than 2000 nucleotides of this genome have been sequenced, including the transforming gene in its entirety.

The genome of MSV is derived from two sources: normal mouse cells and a helper virus, Moloney leukemia virus (MuLV). Analysis of DNA from normal BALB/c mice reveals that the viral transforming gene, called src, is identical to a sequence in normal cells which is called sarc. The normal sequences on either side of the sarc sequence in the cell show no homology with the MuLV sequences in MSV, suggesting that the src sequence in the virus was acquired by a mechanism other than homologous recombination.

In studies to examine this phenomenon, a rarely-occurring variant of MSV was used in which non-MuLV sequences are packaged in the virion. These sequences, which hybridize with a cellular sequence 2000 base pairs outside the 3' end of cellular sarc, are important because they are thought to be involved in the acquisition of sarc by MuLV and may, in fact, regulate recombination.

When single copies of the sarc sequence from normal cells, without flanking

sequences, were placed in other normal cells, the sequence did not cause transformation. However, when the terminal repeat sequences of MuLV provirus were added to the 5' end of the normal sarc sequence and the assay was repeated, efficient transformation was produced. These studies are the first to identify the genetic elements necessary for a normal cell sequence to acquire malignant potential, and are particularly important because they may offer insight into the regulation of genes which can cause cancer.

Cells transformed by sarcoma viruses have been shown to release a family of polypeptide growth hormone-like factors, called sarcoma growth factors (SGFs), into the supernatant fluids in cell cultures. These factors stimulate cell division, compete for epidermal growth factor receptors on the cell surface, and induce normal fibroblasts to express some of the properties of transformed cells, including growth in soft agar. While murine sarcoma virus produes a permanent genetic change in the cells it transforms, SGFs produce only reversible phenotypic changes in normal cells that depend on the continued presence of the factors. These cells can be cycled back and forth between the transformed and normal phenotype without apparent genetic change, by growing them either in the presence or the absence of the SGFs.

Comparison of SGF and Epidermal Growth Factor (EGF) shows that while they share some common properties, they are nevertheless distinctly different. Both interact with the EGF membrane receptor system, are heat-stable peptides requiring disulfide bonds for activity, and are active in nanogram quantities. They differ in that SGF promotes growth where EGF will not, and SGF is produced by sarcoma virus-transformed cells while EGF is not. Antibodies directed against EGF do not recognize SGF, and the isoelectric points of the two growth factors are quite different.

A third category of factors, called transforming growth factors (TGF), is also under investigation. Capable of causing untransformed fibroblasts to assume the transformed phenotype, the TGFs have been found to be released by three different human tumor lines in culture, a rhabdomyosarcoma, a bronchogenic carcinoma, and a metastatic melanoma. In addition, the most anchorage-independent tumor cells (those that can grow in soft agar) released the most TGFs. These proteins are acid- and heat-stable, produce profound morphologic changes in rat and human fibroblasts, and enable normal anchorage-dependent cells to grow in agar. Removal results in a reversion of cell phenotype to normal.

The TGFs are similar in their action to sarcoma growth factor. However, their production is not limited to sarcoma cells, rodent cells, or cells transformed by RNA tumor viruses. In assays comparing growth stimulation by TGFs of mouse, rat, and human fibroblasts in monolayer cultures, there was no evidence that the TGFs produced by human cells are species-specific. They may therefore represent more general effectors of the transformed phenotype.

TGF production by transformed cells and the responses to TGF by normal cells raise the possibility that cells "auto-stimulate" their growth by releasing factors that bind to receptors at the cell surface. Furthermore, continuing production of such factors may be necessary for maintenance of the transformed state. Purification of these factors could lead to the development of specific assays for their presence in body fluids, and elucidation of their

action may suggest means for control of the tranformed phenotype.

Leukemia is the most prevalent form of cancer among children and a significant cancer in adults. For this reason, studies on the leukemia viruses of lower animals are particularly important, since they provide an excellent model system for research. In studies on hematopoietic neoplasms, a valuable system has been developed for studying erythroid cells which were induced to proliferate by three different leukemia viruses. Each virus induces a somewhat different form of erythroleukemia; the genes and gene products of each virus have been identified, and cellular markers and cell culture assays for each form of erythroleukemia are being developed. The next step in this research will be to generate a variety of mutants in the viral genes that cause each form of erythroleukemia and use these to dissect the steps that control the growth and differentiation of blood cell precursors.

An exciting observation has already resulted from these studies. A permanent line of apparently normal erythropoietic mouse stem cells has been established, evidently as a result of infection by one of the erythroleukemia viruses. The cell line is not malignant, and, when injected into mice whose own erythropoietic system has been destroyed by radiation, forms colonies of stem cells which then produce the normal formed elements of the blood.

Intensive research on the viral gene responsible for this growth-promoting effect on stem cells is now underway. The identification of this gene and the protein it codes for is of great importance to biomedical research, since the availability of such a protein may allow the routine growth in culture of unlimited quantities of normal, pluripotent, hematopoietic stem cells for clinical use in humans.

The murine mammary tumor viruses (MMTVs) are unique in being the only viruses known to induce mammary carcinomas, and as such have become important models for the possible induction of human breast cancer. The major envelope glycoprotein of these viruses, which ranges from 52,000 daltons in C3H mice to 55,000 daltons in RIII mice, appears to bear an important relationship to certain antigens in human breast cancer. Using antibodies against the 52,000 dalton glycoprotein (gp 52) in the indirect immunoperoxidase test, investigators found that tumor tissue from 47% of American women with breast cancer who were tested contained MMTV-related antigens which reacted with these antibodies. This reaction was not seen in tissue from benign breast tumors and in only one of 107 carcinomas from other organs, a mucoepidermoid carcinoma of the parotid gland. Among the various types of breast cancer, an invasive carcinoma associated with the intraductal component of breast tissue was found more likely to contain the cross-reacting antigen than either a purely intraductal or purely invasive tumor, and a family history of breast cancer was associated with a high percentage of antigen detection.

Using the indirect immunofluorescence test, other investigators have found MMTV-related human breast tumor antigens in sera of 41% of women with breast carcinoma. In contrast with the study cited in the previous paragraph, however, evidence for the presence of the antigen was also found in the sera of 19% of women with benign breast disease and in 14% of patients with malig-

nancies other than breast carcinoma. The degree of reactivity increased with age and was also elevated in women with ductal carcinoma of the mucinous type. The sera of 42 women with no history of cancer, whose ages ranged from 30 to 80, were negative. The differences in these two studies may be related to the techniques used in analysis and are being investigated.

Because of its obvious potential importance, the human breast tumor antigen is under intense scrutiny and efforts to isolate and purify it are in progress. In one study, a protein weighing 50-80,000 daltons was isolated from human milk and shown to be immunologically related to MMTV gp55. In another study on MMTV gp52, it was found that the immunologically reactive portion of the glycoprotein is a polypeptide with a molecular weight of 8-10,000 daltons, and that within this molecule, a peptide of approximately 2000 daltons is apparently responsible for the cross-reaction with the human breast tumor antigen.

The DNA-containing viruses continue to be of great importance in cancer research. Seroepidemiologic studies have already demonstrated the association of Epstein-Barr virus (EBV) with Burkitt's lymphoma (BL) and nasopharyngeal carcinoma, and markers from this virus are being used as aids to the diagnosis and prognosis of both diseases. Studies are continuing to determine whether there is a portion of EBV DNA common to the various strains of the virus which have been derived from BL patients. Thus far, transforming strains from infectious mononuclosis and BL have demonstrated considerable homogeneity in their DNA, with only slight variations in the size of certain fragments. Physical maps of the various DNA's are being prepared for a more detailed study.

Progress is also taking place in identifying the transforming proteins coded by DNA viruses. In the case of simian virus 40 (SV 40), an antigen (T antigen), appearing in the nucleus of infected cells and specified by one of the three genes of the virus, may be involved both in transformation and in regulation of viral DNA replication and transcription. The recent finding of protein kinase activity in SV40 T antigen provides a strong basis for determining the targets of this enzymatic action.

Papovaviruses of the SV40-polyoma subgroup have been isolated from primates, rabbits, rats, and mice. As a rule, antiserum against intact virions of a given member of the subgroup shows marked specificity for that virus, and no other. Only between the primate papovaviruses, BK, JC, and SV40, has slight cross-reactivity been shown. However, in a study using antisera against disrupted virions, it was found that there is a viral polypeptide (VP1) within the virus particles which is antigenically common to all the members of the SV40-polyoma subgroup. This suggests that the subgroup has a common evolutionary origin and that VP1 may be useful in identifying new viruses of this subgroup.

The hr-t gene of polyoma virus is important in cell transformation and in coding for both small and middle T antigens of the virus. It appears to govern all the cellular changes occurring during transformation, including changes in the cytoskeleton and cell surface, gross morphological changes, loss of growth control, and tumorigenicity. Studies are now underway to

elucidate the individual functions of small and middle T antigens, and to determine whether both proteins are necessary for all of the expressions of the hr-t gene.

As the review of research in this section makes clear, there has been a dramatic increase in our knowledge of the transforming genes (collectively called "onc" genes) carried by retroviruses and of the gene products of some of them. These viral "onc" genes are derived by recombination from normal cellular genes, which are themselves highly conserved in all vertebrate species; indeed, they have been detected in human DNA. The ubiquity of these genes suggests that, under ordinary conditions, they may serve a normal function which, aberrantly or inappropriately expressed, may cause malignancy.

To date, approximately 8-10 distinct "onc" genes have been recognized, various ones of which are specific for fibroblasts, B lymphocytes, myeloblasts, and erythroblasts. Many of these have already been cloned in recombinant DNA host-vector systems, and others are in the process of being cloned. Consequently, it is now possible to prepare pure, well-defined DNA probes for all of these important genes, and to determine whether any of the genes are expressed in human tumors. This is important because it might lead to new ways to characterize and classify human tumors as well as possibly providing tests for differential diagnosis. Moreover, if a particular form of cancer were found consistently to show a high level of expression of a particular "onc" gene, it would represent a major advance in understanding the mechanism of tumorigenesis of that particular tumor. Whatever the specific results of these new discoveries, however, it seems clear that they offer an unparalleled opportunity for basic molecular tumor virology to make major contributions to resolving the problem of human cancer.

Chemical and Physical Carcinogenesis

Interest in chemical carcinogenesis continues to be high, as new, potentially carcinogenic chemicals are found in the air, the water, and the food consumed by millions of people in industrialized societies throughout the world. In addition to industrial chemicals, however, there must be considered those chemicals to which people are exposed by lifestyle. At present, the most significant of these are found in tobacco smoke, but increasing evidence suggests that many forms of cancer other than that of the lung may be linked to chemicals, as yet unidentified, which are naturally present in food or are created during its preparation.

Central to this is the importance of learning precisely how certain chemicals cause cancer. It appears that cancer is a disease in which genetic information and/or expression are altered, and that this may be induced by exogenous or endogenous factors which result in altered patterns of gene control. As in biological carcinogenesis, therefore, one of the primary aims of chemical carcinogenesis is the elucidation of the mechanisms of transformation.

The past year has seen a number of promising developments in this area. Attempts to detect, isolate, and identify the cellular genes involved in the expression of the transformed phenotype are being made; one approach is to detect the cellular genes which have the capacity to induce or suppress the transformed phenotype. Cells resulting from the hybridization of normal human diploid fibroblasts and chemically transformed counterparts showed a transformed phenotype in vitro; in addition, exposing normal human diploid fibroblasts to DNA extracted from ouabain-resistant human fibroblasts resulted in the appearance of ouabain-resistant cells. These results indicate that DNA-mediated transfer of the transformed phenotype may be possible, and that the cellular genes responsible for the expression of the transformed phenotype can be identified by using this, or a similar, cell system.

During evolution, humans and their progenitors have been exposed to foreign chemical compounds, including carcinogens, and have developed systems for their detoxification and elimination. Some of these systems, however, are also capable of metabolizing certain chemicals to much more carcinogenic forms. Human lymphocytes and monocytes have been shown to exhibit mixed function oxidase activity, a property which in many tissues and species is responsible for the activation of polycyclic hydrocarbon carcinogens as well as their inactivation. The levels of this activity vary among individuals and may be influenced by a variety of factors such as drugs, pesticides, carcinogens, age, genetic makeup, and nutritional state.

Excellent model systems have been developed to study the problem of carcinogen metabolism by various tissues. Human bronchus, colon and esophagus in culture have been found to metabolize various carcinogens or procarcinogens found in the environment and/or tobacco smoke, including polynuclear aromatic hydrocarbons, N-nitrosamines, hydrazines, and myco-

toxins. Importantly, metabolic pathways and carcinogen-DNA adducts were generally similar among various species, including humans. However, wide quantitative differences in the binding of carcinogens to DNA were found among different people. In addition, quantitative and, in some cases, qualitative differences have been found among human tissues with regard to the organ specificity of chemical procarcinogens. As the enzymes and pathways responsible for these differences are characterized and sensitive methods for their assays are developed, it may be possible to characterize an individual's specific carcinogen metabolism; this, in turn, could lead to an understanding of individual susceptibility to cancer caused by environmental procarcinogens.

Since most chemical carcinogens are also mutagens, assays of mutagenesis are useful as screening tests for chemical carcinogens. In many of these assays, promutagens (procarcinogens) are activated by microsomal preparations from either experimental animals or from human tissues. However, since metabolic activation of procarcinogens by microsomal preparations may not accurately reflect metabolic activation within intact cells, a mutagenesis assay has been developed in which promutagens are metabolically activated by explants of human target tissues such as bronchus. When these explants are cocultivated with V-79 Chinese hamster cells (which do not activate procarcinogens), any activation by the human tissue is reflected by mutation of the hamster cells. This same phenomenon is also seen when human pulmonary alveolar macrophages are co-cultivated with V-79 cells, suggesting that macrophages and bronchial epithelium may act in concert metabolically to activate inhaled chemical carcinogens. Such human tissue- and cell- mediated tests, in addition to focusing attention on the possible interactions between the non-target and target cells of carcinogens during carcinogenesis, also have potential as screening tests for chemical carcinogens and as assays to help determine oncogenic susceptibility, both among individuals and among different target tissues in a single individual.

While the study of chemical transformation of mammalian fibroblasts in culture has contributed significantly to our knowledge of chemical carcinogenesis, the study of epithelial cell transformation may offer distinct advantages not found in established fibroblast systems. For example, because of their high susceptibility to carcinogenic polycyclic hydrocarbons, the epithelial cells of the rat mammary gland provide an excellent model for the study of *in vitro* transformation of a differentiated cell. Initial studies have established that such cells possess the enzymes necessary to metabolize carcinogenic hydrocarbons. Furthermore, after treatment by carcinogens *in vitro*, the mammary epithelial cells display anchorage-independent growth, a phenotypic change often correlated with the ability to form tumors *in vivo*. These studies, and others to follow using this system, will provide an essential framework for the investigation of epithelial transformation *in vitro*.

Since, in daily life, most of the population is exposed to a diversity of potentially carcinogenic factors, it is important to determine the extent to which one carcinogenic agent influences the frequency of transformation by another agent from a different category of carcinogens. The study of alteration of transformation frequency by exposure to multiple agents, both

carcinogens and noncarcinogens, is important not only to understanding the mechanisms of transformation, but also because combinations of various agents may be responsible for increased risk of human cancer. One critical finding in this area is that pretreatment of cells by X-irradiation, or by an alkylating agent such as methylmethane sulfonate (MMS), results in an increase in the number of transformations by a variety of carcinogens. Under the experimental conditions used, the X-ray pretreatment alone produced no transformation and the MMS produced only rare transformations. Another significant finding of this study was that the enhancement of transformation by X-rays or alkylating agents is independent of whether the chemical carcinogen used is a polycyclic hydrocarbon, another alkylating agent, or a compound whose effects mimic that of UV irradiation.

In studies to investigate this phenomenon further, the effect of both X- and UV-irradiation on transformation was probed. Several important conclusions were reached: first, in the case of transformation following irradiation pretreatment, the target for transformation is significantly larger than a single locus mutation; second, the enhancement of transformation by X-ray does not result from a change in either the rate of excision of pyrimidine dimers or in post-replication repair; and third, the results suggest that other repair systems, such as the recovery of nascent DNA strands to normal size, may be important to cell survival or, functioning inappropriately, to transformation and its enhancement.

Human exposure to the carcinogen benzo(a)pyrene (BP) is ubiquitous and is particularly significant in cigarette smoke, heavily polluted air, drinking water, and smoked foods. Exposures are exceptionally high for coke oven workers, gas works operators, and asphalters whose occupations have been associated with increased cancer risk. Thus, any chemical which can block carcinogenesis by BP would be of great practical interest. One such chemical which is highly effective is butylated hydroxyanisole (BHA), one of the most versatile chemopreventive agents known. This compound inhibits neoplasia at many organ sites induced by almost every category of chemical carcinogen. Previous studies have indicated that an important mechanism of this inhibition, in the case of BP carcinogenesis, may be the capacity of BHA to cause enhanced detoxification by alteration of the microsomal mixed function oxidase system. Recent investigations have now shown that BHA increases the activities of two major conjugating systems of drug detoxification, cytosolic glutathione-S-transferase activity and microsomal glucuronyl transferase. Further, BHA produces a rise in the levels of acid-soluble sulfhydryl compounds, reduces the levels of urinary mutagenic BP metabolites, and reduces the mutagenic activities of BP. Liver microsomal epoxide hydratase activity is also markedly enhanced. Altogether, these studies have demonstrated that BHA increases the activities of 6 hepatic microsomal enzymes, at least three hepatic cytoplasmic enzymes, as well as promoting transferase and hydratase levels in several extrahepatic tissues.

Recent findings concerning the metabolism of benzo(a)pyrene to mutagenic and carcinogenic metabolites have greatly aided a study of the mechanism of inhibition of BP carcinogenesis by BHA. In this investigation, the effect of BHA on both BP and (+)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene-induced neoplasia was studied following intragastric intubation of these compounds. Butylated hydroxyanisole was found to inhibit both BP-induced neoplasia of the forestomach and BP 7,8-dihydrodiol-induced neoplasia of the forestomach, lungs, and lymphoid tissues. Based on current data that BP 7,8-dihydrodiol is a proximate carcinogen and BP 7,8-diol-9,10-epoxide is an ultimate carcinogen, these results suggest that at least one mechanism for the inhibitory effect of BHA on BP carcinogenesis involves events which occur subsequent to the formation of BP 7,8-dihydrodiol.

Research into the mechanisms of inhibition of carcinogenesis is important because it offers the ultimate promise of tailoring inhibitors which can be taken by people who cannot, or will not, avoid exposure to specific carcinogens. Such research, which falls within the classification of chemoprevention, seeks means by which the process of transformation can be prevented or reversed. Some of the most promising agents being investigated in these studies are the retinoids, analogues of vitamin A, which are being examined for their ability to prevent the development of invasive cancer while the disease is still in its early stages.

The ability of both natural and synthetic retinoids to inhibit two-stage mouse skin tumorigenesis correlates with their ability to specifically inhibit the induction of ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine biosynthesis. This is important because the induction of polyamine biosynthesis by tumor promoters, such as the phorbol esters, may be one obligatory event in the promotion of skin carcinogenesis. However, neither the induction of increased soluble protein synthesis nor the induction of S-adenosylmethionine decarboxylase (SAMDC), the second enzyme in polyamine biosynthesis, is affected by retinoids. Those retinoids which inhibit tumor promotion and the induction of ODC activity also inhibit the accumulation of the polyamine putrescine, the product of ODC action, but not the levels of spermidine and spermine, which depend upon the action of SAMDC.

Some of the difficulties with using retinoids in animal studies have been associated with their pattern of tissue distribution. It has been observed that alteration of a retinoid's structure, such as changing a terminal carboxylic acid group to a less polar derivative (an ether, an amide, or a hydrocarbon) may lessen the molecule's toxicity and change its tissue distribution as well. As a result of work of this kind, a compilation of structure-activity relationships of several hundred retinoids has been completed. This compilation will allow selection of new retinoids for further studies of cancer prevention in animals, particularly cancer of the bladder and breast, with the goal of developing practical agents for prevention of these malignancies in humans.

Increased emphasis is being given to the search for inhibitors that block the formation of N-nitrosamines from nitrites and amines,compounds which are widely found in the environment and in food products. Ascorbic acid (vitamin C) has received considerable attention because of its suggested role in cancer prevention. It has also been implicated as a possible inhibitor of nitrosamine formation, having been shown to inhibit formation of many nitrosamines, including dimethylnitrosamine, methylnitrosourea, nitrosomorpholine and mononitrosopiperazine. However, it is not completely effective in blocking formation of N-methyl-N-nitrosoaniline.

The reaction of nitrite ion with ascorbic acid and its effect on the rate of nitrosation of secondary amines have been investigated. Unfortunately, ascorbic acid does not show uniform behavior. It accelerates the nitrosation of N-methylaniline between pH 1.00 and pH 1.95. It allows nitrosation of diphenylamine and iminodiacetonitrile, but inhibits nitrosation of secondary amines such as dimethylamine, diethylamine, proline, hydroxyproline, N-methylaminoacetonitrile, N-methylaminopropionitrile, and sarcosine. The nitrosating agent generated by the reaction between ascorbic acid and nitrite ion appears to be oxyhyponitrite. Since nitrosamines have the ability to transnitrosate under appropriate conditions, the addition of ascorbic acid to a commercial product containing a variety of unknown amines may not have the desired effect. Ascorbic acid might slow down the formation of some nitrosamines, but in contrast, it might accelerate formation of others which, although less potent carcinogens, might generate more potent ones by transnitrosation. This possibility, still hypothetical, is under examination.

Field Studies and Statistics Program

With rising public interest in the environmental causes of cancer and in the contributions that epidemiology can make to understanding the etiology and prevention of cancer, the Field Studies and Statistics Program plans a comprehensive, flexible, and balanced program to generate fresh ideas and help settle important questions in cancer epidemiology and biometry. During the past year, the Program has conducted studies on the etiology and natural history of cancer, end results, clinical trials, and preventive measures.

To provide a systematic means for identifying geographic variation and clustering, the Program has analyzed U.S. cancer mortality on a county level and prepared computer-generated color maps showing the incidence of cancer in 35 sites of the body. First, an atlas of cancer mortality was published for the white population, followed by a companion atlas for the non-white population covering the years 1950-69. At present maps are being prepared on cancer mortality for the time period 1970-75. Maps for non-neoplastic diseases have also been prepared, emphasizing conditions that predispose to cancer or share etiologic factors. The Program has actively pursued the clues derived from the cancer maps, using a stepwise approach of correlation and analytic studies.

A number of occupational studies were reported during the past year. Preliminary results from a survey of non-agricultural pesticide applicators revealed an excess of deaths from lung cancer, particularly among workers licensed for ten or more years. A proportionate mortality study of Government Printing Office employees revealed excesses of multiple myeloma among composing room workers possibly exposed to benzene. A proportionate mortality study of workers in the metal polishing and plating industry showed excesses of esophageal and liver cancers, possibly due to exposures to metal fumes, solvents, and strong acid or alkaline substances. A study of causes of death among veterinarians revealed significant increases of leukemia and Hodgkin's disease, and cancers of the brain, liver, and skin. The mortality from cancer was greatest among veterinarians in clinical practice, raising the possibility that specific work exposures (e.g., medical x-rays, animal viruses) may be carcinogenic. In addition, analysis of occupations recorded on death certificates from Nebraska (1957-74) revealed an increased risk of leukemia among farmers, especially from heavy corn producing counties.

Several large-scale mortality surveys of industrial groups were also carried out this year. A study of active and retired employees of a pharmaceutical company indicated excesses of respiratory cancer and leukemia among female production workers, and respiratory cancer among male maintenance workers. These associations could not be traced to specific chemical exposures, but provide leads to further studies. An evaluation of mortality among members of the Oil, Chemical, and Atomic Workers International Union (OCAW) indicated elevated frequencies of cancers of the stomach, kidney, brain, leukemia, and multiple myeloma among petroleum refinery and petrochemical workers who were OCAW members for at least ten years. Mortality ratios for cancers of the stomach, brain, and lung were especially high in specific plants, where more definitive studies are planned. A proportionate analysis of 202 deaths among aircraft spray painters exposed to zinc chromate paints revealed an excess of lung cancer, consistent with the risks seen in chromium manufacturing

processes. A survey of iron foundry workers exposed to polycyclic hydrocarbons revealed excess mortality from cancers of the respiratory and digestive systems.

As pressure mounts to identify the health effects of low-level exposures to ionizing radiation, large-scale studies of irradiated populations have been undertaken. These include studies of over 100,000 cervical cancer patients treated with radium implants; 20,000 former tuberculosis patients who received multiple fluoroscopies; 3500 children exposed to head and neck irradiation during childhood; and 80,000 survivors of the atomic bombings in Hiroshima and Nagasaki. Radiation studies of latent period, dose response, interactions with host factors (e.g., age), and other risk factors have basic implications for theories of carcinogenesis, and particular attention is paid to these parameters during the course of the investigations.

To characterize the risk of breast cancer from radiation, data on women in three of the aforementioned groups were analyzed. The exposed groups included tuberculosis patients who had multiple fluoroscopic examinations of the chest, mastitis patients given radiotherapy, and A-bomb survivors. The findings suggest the following: the risk of breast cancer is greatest in persons exposed as adolescents, although exposure at all ages carries some risk; the dose-effect relationship is consistent with linearity; fractionation does not appear to diminish risk nor does time since exposure, even after 45 years of observation; and the interval between exposure and clinical appearance of radiogenic breast cancer is mediated by age-related factors, perhaps hormonal, but is unrelated to dose.

Findings from the continued followup of women exposed to multiple fluoroscopic chest examinations in conjunction with pneumothorax treatment of tuberculosis reaffirm that repeated, relatively low doses of radiation pose some future risk of breast cancer, that the risk may be cumulative, and that a woman's life-time risk of breast cancer may be determined in large part during her reproductive years. Exposures around menarche and during first pregnancy appear especially hazardous, indicating that proliferating breast tissue may be particularly sensitive to the carcinogenic effects of radiation. However, no excess of total cancer deaths, leukemia, or lung cancer was seen among fluoroscopically-examined women.

Medical drugs have been generally thought to contribute only slightly to the overall risk of cancer, but recent findings suggest that the hazard may be much greater than anticipated. This year, several projects were undertaken to evaluate the carcinogenic effects of estrogen compounds. A case-control study of breast cancer using the Breast Cancer Detection Demonstration Project revealed increases in relative risk, primarily in women with a surgical menopause. Estrogens appeared to obliterate the protective effect normally associated with oophorectomy, and produced an increased risk with extended use. Nearly a four-fold excess risk was seen for cancers detected after the initial screening among users of ten or more years. The highest risks were seen when hormones were used in the presence of known risk factors, including nulliparity, family history of breast cancer, and benign breast disease. Another study involved 345 women with breast cancer and 611 controls, all members of a prepaid health plan. There was significant excess risk among women who had ever used conjugated estrogens; a dose-response rela

tionship was seen with three different measures of dose, rising to two-fold following long-term use.

Interest in dietary factors associated with the etiology of cancer continues to increase. Several case-control studies of populations in high cancer-risk areas have probed for nutritional factors that might positively or negatively affect the incidence of malignancies. In two rural counties in Nebraska with a cluster of colon cancer, the risk was primarily among people of Czech ancestry and was associated with a high intake of fat and dairy products. In a case-control study of esophageal cancer among blacks in Washington, D.C., alcohol consumption was the primary risk factor and provided approximately 40% of the daily caloric requirement. After controlling for alcohol intake, the consumption of fruit and vegetables containing vitamins A and C was inversely related to the risk of esophageal cancer. The role of micronutrient deficiency is also being evaluated in case control studies of lung cancer, and the influence of fat intake is being explored in breast cancer.

In further studies of alcoholism in collaboration with the Medical Follow-up Agency, a mortality survey of U.S. Army servicemen hospitalized for chronic alcoholism in 1944-45 revealed an excess of several causes of death, including cancers of the buccal cavity, pharynx, larynx, and esophagus . The findings support previous studies suggesting that alcohol potentiates the carcinogenic effect of tobacco smoke, perhaps through nutritional deficiencies or co-carcinogens that may be present in alcoholic beverages.

Epidemiologic patterns of cancer suggest that the environment may contribute more to cancer risk than do genetic factors. Inherited suceptibility and interactions with environmental influences are usually too subtle or complex for detection by ordinary epidemiologic means. However, when susceptibility is conspicuous, as in cancer-prone families, research opportunities are provided that may clarify the role of genetic and environmental factors in carcinogenesis. This research strategy is illustrated by the evaluation of familial melanoma and its relation to the dysplastic nevus syndrome, first described by Program staff in 1978. Of 70 melanoma-prone families, nearly all have precursor moles. Reports this year helped to further charcterize the mole syndrome, its occurrence in some patients with sporadic melanoma, and its treatment by topical chemotherapy. Over 350 members of 13 high-risk families have been examined, and biological specimens collected for a battery of laboratory studies, including immunologic investigations. Identification of this syndrome has enabled the diagnosis and removal of early-stage melanoma, and a comprehensive educational program has been developed for families and physicians.

Because of the drugs routinely taken by renal transplant patients to suppress the immune response, special attention has been given to these individuals to detect links between immunosuppression and cancer. An updated analysis of 16,000 renal transplant recipients reported to the American College of Surgeons has revealed a 32-fold excess of non-Hodgkin's lymphoma, mainly histiocytic lymphoma (about 150 times the expected rate for this particular malignancy). The risk of other cancers was increased two-fold, but did not affect all tumors across-theboard, as might be expected by the immuno-surveillance hypothesis. Instead, the excess was limited to particular

forms of cancer, including hepatobiliary neoplasms, lung adenocarcinoma, urinary tract tumors, soft-tissue sarcomas, melanomas, and squamous cell carcinomas of the skin. Although oncogenic viruses may be involved in post-transplant lymphoma, there was no evidence of time-space clustering or higher incidence in transplant recipients having contact with lymphoma patients in the induction period. These findings do not exclude a role for viruses in post-transplant lymphomas, since malignant transformation from a latent viral infection would not be reflected in clustering.

The Surveillance, Epidemiology, and End Results (SEER) Program obtains cancer incidence and patient survival data through a group of eleven population-based cancer registries covering all cancers diagnosed in the populations of five entire states (Connecticut, Hawaii, Iowa, New Mexico, and Utah), five metropolitan areas (Atlanta, Detroit, New Orleans, San Francisco, and Seattle) and the commonwealth of Puerto Rico. The first report on cancer incidence and mortality, covering this entire program, was published in October 1978. A monograph of over 1,000 pages presenting detailed data, area by area, for the period 1973-1977 has been submitted for publication, thereby making a set of basic data available to cancer reseachers as well as to those concerned with various aspects of the cancer problem.

The data for 1973-77 have been used to make comparisons among the various ethnic groups within the eleven SEER area populations. When ranked by incidence rates per 100,000 population for all sites combined, both Hawaiian males (465.0) and Hawaiian females (408.5) had the highest rates in their respective sex groups. Among the males, Blacks (454.3) were second and Whites (371.6) third while among females, Whites (301.2) were second and Blacks (288.7) third. For individual sites, however, the picture varied considerably. The Hawaiians in Hawaii and the Hawaii-Japanese had by far the highest stomach cancer rates among males (51.4 and 47.3 respectively) and the whites had the lowest (12.7). For colon cancer among males, on the other hand, the rates were highest among six groups (ranging from 34.3 to 39.6)--Whites, Blacks, San Francisco-Chinese, Hawaii-Chinese, San Francisco-Japanese and Hawaii Japanese. The lung cancer rates were highest among Hawaiians for both males and females (116.9 and 50.7 respectively). They were next highest among Black males (110.0) and White males (76.4). Among females, lung cancer incidence was also relatively high among the San Francisco-Chinese (33.1) and the Hawaii-Chinese (29.8). Examination of the primary site distribution for each ethnic group reveals that for males, lung was the most frequent site of cancer among Blacks, Hawaiians, and Whites. Among the Japanese, Chinese, and Filipinos, colon and rectum was the leading site, and among the Puerto Rican-Spanish, the New Mexico-Spanish and the American Indian, prostate was the most frequent site. For females, breast was the most frequent site for every ethnic group, but for the American Indian cervix was equally as frequent, with gallbladder following closely behind. Detailed analysis of these data for ethnic groups will provide a basis for the formulation of specific hypotheses for further study.

SUMMARY REPORT

FREDERICK CANCER RESEARCH CENTER

Introduction: Work conducted by the Contractor on behalf of the NCI Division of Cancer Cause and Prevention at the Frederick Cancer Research Center consists of three major efforts: the Biological Carcinogenesis Program, the Chemical Carcinogenesis Program and the NCI Carcinogenesis Intramural Program.

In their original form in the early- to mid-1970s, precepts regarding the conduct of the three programs were such that each of these embraced a series of loosely joined tasks or projects designed to furnish effort toward the accomplishment of a number of relatively independent NCI research or resource objectives. As scientific accomplishments accrued and research expertise was acquired, the semi-dichotomous nature of the various efforts gave way, becoming highly amenable to a blending into single overall interrelated research programs embracing a variety of scientific disciplines. This is amply evidenced in the evolvements of the current Biological Carcinogenesis (BC) and Chemical Carcinogenesis (CG) Programs. Moreover, major portions of work by on-site NCI investigators are now conducted as comprehensive parts of overall DCCP program goals at FCRC, while meeting research obligations at NCI, Bethesda.

Biological Carcinogenesis Program. The overall goal of this Program is to conduct investigations on the complex relationships between host cells and oncogenic and suspected oncogenic viruses. Studies are performed on multiple models of virus-cell interaction employing acute and subacute agents considered likely to be applicable to human cancer to: (i) determine whether viruses, viral gene sequences, and/or their products comparable to those known to induce cancers of laboratory and domestic animals are associated with, indicative of, or in fact instrumental either alone or as carcinogens in inducing the development of human cancers, (ii) define the character and mode of action of virus and/or viral components in their relationships to tumor formation, and (iii) to devise therapeutic and preventive measures for their control. Functional laboratory elements within this DCCP/FCRC program will conduct basic, exploratory and applied research on programs that are or could be vital to the Cancer Program. Moreover, as a highly important resource activity, a diminished but adequate targeted effort will be maintained to furnish critically needed biologic working materials for programs both within and outside FCRC. The six major laboratory elements constituting the framework within which scientific effort in the Biological Carcinogenesis Program will be conducted are the following: Molecular Biology of Retroviruses, Immunochemistry, Molecular Biology of Carcinogenesis, Primate Virus Immunobiology, Molecular Mechanisms in T-Cell Leukemogenesis and Viral Resources.

Chemical Carcinogenesis Program. This Program has as a primary objective the development of a unique national resource capable of initiating and evaluating a broad range of approaches to effectively identify human carcinogens. The responsibility of chemical carcinogenesis research is to investigate factors that contribute to or result in the induction of cancer in humans after

exposure to chemicals, and to investigate means of preventing subsequent development of cancer; the influence of both extrinsic and intrinsic factors are included in the investigations. To do this requires a coordinated interdisciplinary approach which consciously emphasizes the integration of basic and applied research. Broadly, the areas of research being supported are: (i) development of methods and conduct of testing for the purpose of identifying environmental chemicals that are carcinogenic or cocarcinogenic; (ii) investigation of host-chemical interactions with the ultimate aim of being able to alter host susceptibility and response after exposure; and (iii) attempts to elucidate mechanisms of carcinogenesis on the premise that an understanding of the process will permit the manipulation of factors which will block or reverse tumor formation. The nine major laboratory elements constituting the framework within which scientific effort in the Chemical Carcinogenesis Program will be conducted are the following: Mechanisms of Nitrosamine Carcinogenesis, Chemistry of Carcinogens, Molecular Aspects of Chemical Carcinogenesis, Cellular Aspects of Chemical Carcinogenesis, In Vitro Carcinogenesis, Chemical Synthesis and Analysis, Microbial Mutagenesis Screening, In Vivo Carcinogenesis Assessment, and Pathology and Histotechnology.

During the past year two programs were phased out: Biology of Type C Viruses in the Biological Carcinogenesis Program, and Endogenous Formation of Carcinogens in the Chemical Carcinogenesis Program, with portions of each effort either redirected or held in abeyance pending further program judgements by NCI. Also of importance is the transfer of the Mechanisms of T-Cell Leukemogenesis project from the Cancer Biology Program (DCBD) to the Biological Carcinogenesis Program (DCCP) where it has a much more likely "fit" with other work being conducted. These program alterations represent definite steps that will sharpen and strengthen even more the total DCCP/FCRC research effort.

Carcinogenesis Intramural Program. As an administratively separate and distinct effort, the Contractor furnishes necessary scientific and support services involving appropriate technical expertise and materials and supplies on behalf of a number of on-site research efforts conducted by senior NCI/DCCP scientists at FCRC. At present, NCI/DCCP intramural programs to be accommodated at FCRC consist of but are not necessarily limited to the following, subject to change at the discretion of the government: Viral Genetics Section, Laboratory of Cellular and Molecular Biology; Viral Immunology Section, Laboratory of Cellular and Molecular Biology; Bio-molecular Oncology Section, Laboratory of Cellular and Molecular Biology; Viral Immunotherapeutic Studies, Laboratory of Cellular and Molecular Biology; and Microbiology Section, Laboratory of Molecular Virology.

Types and kinds of Contractor support required for NCI intramural effort consist of technical expertise in a wide range of scientific disciplines including but not necessarily limited to: chemistry, biochemistry, immunochemistry, immunology, serology, virology, electronmicroscopy, biophysics, cell biology, and cell culture. Also needed are Contractor services to furnish quality control testing for mycoplasma and/or other adventitious agents, glassware and media preparation, animal care, janitorial services, and some administrative, including clerical, assistance. Possible part time needs may include

histotechnological and veterinary science services. Additionally, there are requirements for biological resources such as laboratory animals, and purified viruses and/or viral products. Not all types of technical expertise, services, and resources given above are required for each of the intramural work segments listed. Support needed for any changes or additions in intramural effort will be identified subsequently as intramural research objectives at FCRC are developed.

DCCP/FCRC Program Administration: With approval of the Director DCCP, all Contractor activities performed at FCRC on behalf of the Division are the responsibility of the NCI Scientific Coordinator for DCCP in residence at Frederick. Functioning as Assistant Project Officer, FCRC, the Coordinator designs and interprets the workscopes and assures attainment of scientific goals and objectives under the contract mechanism. The DCCP Coordinator also directs the Contractor so as to insure appropriate personnel and physical resources support applicable to NCI senior investigators conducting their research at Frederick.

All work conducted within the DCCP area at FCRC is reviewed formally on a continuing basis for the purpose of attaining and maintaining excellence of programs that have been established and to insure rapid responses of DCCP/FCRC operations to needs related to Division and NCI Program objectives.

Recommendations that have played a highly significant role in the ultimate determinations of DCCP/FCRC Programs have been provided by a number of peer review and advisory groups comprised of committees consisting of recognized outside experts. Such recommendations stem from scientific reviews that are conducted annually as a minimum, the most recent being by the DCCP Board of Scientific Counsellors, which performed an in-depth evaluation of DCCP/Contractor efforts the latter part of September 1979. Added to that, the National Cancer Advisory Board met in May to consider, on site, all FCRC activities including those supported by DCCP. In-house recommendations are obtained on an as needed basis from NCI staff who provide specialized individual or ad hoc committee assistance. Additionally, there are a number of routine review and evaluative processes conducted by NCI staff that take place at monthly and semi-annual intervals. These are accomplished in conjunction with a number of specially designated NCI Monitors and Coordinators whose judgements are applied toward determination of the Contractor's fee by the NCI/FCRC Award Fee Evaluation Board.

More specifically, lines along which Biological and Chemical Carcinogenesis Programs proceeded during the past year are as follows:

Molecular Biology of Retroviruses. Using all contemporary techniques of nucleic acid chemistry, determine role of retroviruses in normal and tumorigenic processes of the host species. Objectives are to: characterize viral genomes responsible for oncogenic properties; study molecular mechanisms of replication; map viral genomes to identify active fragments; determine relatedness among viruses and primate cell DNA; and develop cloning techniques to generate probes for screening human DNA.

Immunochemistry. Conduct immunological characterization and amino acid sequence analysis of retroviruses. Objectives are to study: structural similarities and phylogenetic relationships; role of viral polypeptides in assembly, maturation, transformation, and oncogenicity; cleavage of precursor polyproteins and processing enzymes; structure of immunologic determinants; synthesis of peptides corresponding to antigenic regions of virus proteins for use as immunologic probes and potential vaccines; analyses of virus-related proteins and development of immunochemical techniques for entire Biological Carcinogenesis Program.

Molecular Biology of Carcinogenesis. Conduct investigation on mammary tumor viruses, which are unique in that they represent the only ones known to induce mammary carcinomas; are important models for solid tumors of epithelial origin. Objectives are to study: nature and control of expression of MMTV proteins, surface antigens, and other viral components; analysis of MMTV genomes for oncogenic potential; interaction of MMTVs with their hosts: cellular and humoral immune responses; comparisons of endogenous and exogenous MMTVs for immunology, genetic relatedness, tumor induction; and develop and use monoclonal probes for studies in human systems.

Primate Virus Immunobiology. Develop primate models for studies on immunology and pathogenesis of lymphomas and leukemias. Objectives are to study: role of EBV-like Herpesvirus papio in spontaneous lymphoma in baboons; studies of herpesvirus isolates from chimpanzees, orangutans, and gorillas; pathogenesis and immunology of T-cell lymphomas induced by Herpesvirus saimiri in monkeys; and mechanisms of EBV-induced malignant transformation of cultured cells.

Mechanisms of T-Cell Leukemogenesis. Investigate etiology and molecular events in induction of leukemias in murine models. Objectives are to study: T-cell differentiation and function including T-cell antigen specificity; role of type C viruses and chronic immune stimulation in induction of T-cell leukemias; and comparative mechanisms of leukemia induction by radiation, hydrocarbons, urethan, and estrogens in mice.

Viral Resources. To provide facilities and conduct a major resource effort for the large-scale production of viruses or viral components for use within the FCRC and for distribution through the NCI DCCP's Biological Carcinogenesis Branch Research Resources Program, by providing high quality retrovirus concentrates and conducting developmental research for eliciting and/or optimizing virus expression and recovery, and pursuing product improvement studies.

Mechanisms of Nitrosamine Carcinogenesis. Investigate carcinogenic potential of N-nitroso chemicals derived from environmental amino compounds and nitrite. Objectives include: mechanisms of formation of N-nitroso compounds; feeding of amines with nitrosating agents; investigate structure - neoplastic activity relationships; study potency and organ/species specificity of N-nitroso compounds; distribution of N-nitroso compounds and their precursors in the environment; and synthesis of N-nitroso compounds with known structure to determine metabolic pathways toward carcinogenesis (or detoxication) in various organs/species.

Chemistry of Carcinogens. Elucidate mechanisms of carcinogenesis as related to chemical structure. Objectives are to: study how nitrogenous organic chemicals such as nitrosamines, triazines, hydrazines and related substances are activated to become carcinogens; suggest ways in which active intermediates interact with cellular components; and refine information on known or suspected biochemical mechanisms of carcinogen formation.

Molecular Aspects of Chemical Carcinogenesis. Elucidate molecular aspects of carcinogenic process related to interaction between carcinogen and/or its metabolites and host DNA. Objectives are to study reaction of alkylating agents with nucleic acid constituents to understand site selectivity; studies on properties of DMBA-DNA adducts to demonstrate metabolic pathways and factors affecting DMBA-deoxyribonucleoside excision; and compare different DMBA-DNA adducts and operative metabolic routes in vitro and in vivo and influence of carcinogen dose on these activities.

Cellular Aspects of Carcinogenesis. Elucidation of mechanisms in chemical carcinogenesis emphasizing transformation of epithelial cells. Objectives are to develop assays for in vitro transformation of liver epithelial cells (including human liver cells); investigate sequence of events in epithelial cell transformation; and identify the target cells for chemical carcinogens in liver.

Chemical Synthesis and Analysis. Preparation and characterization of a variety of chemical carcinogens and development of analytical expertise to support DCCP/FCRC programs. Functions include efforts to: synthesize and purify carcinogens primarily in support of chemical carcinogenesis projects; develop and use analytical methods to characterize carcinogens and identify impurities; and develop new methods for synthesis and analysis of carcinogens.

Ultrastructural Studies. Conduct electron microscopy studies to identify chemical reactions of the inducing chemical agent and characterizing the biological response of the affected cells. This includes work to: identify morphologically those cells that are targets for chemical carcinogens; identify morphological basis for early detection and diagnosis of precancerous lesions; and contribute to understanding mechanisms of action of nitrosamines and/or their precursors.

In Vitro Carcinogenesis. Development of short-term in vitro carcinogenesis bioassay systems for detecting carcinogens that may be hazardous to man and use these systems for studying mechanisms of chemical carcinogenesis, to provide information for preventing, inhibiting or reversing carcinogenic process. Objectives are to: study various metabolic activation, including enzyme, systems from various animal species that affect cell transformation; determine endpoints for activity such as elevation of aryl hydrocarbon hydroxylase and epoxide hydrase; study induction of metabolic activation enzymes in target cells in vitro or in donors in vivo; and examine cell cultural factors that enhance transformation response.

Microbial Mutagenesis Screening. Provide a microbial mutagenesis testing service in conjunction with chemical carcinogenesis (and other) program elements for prescreening evaluation of mutagenic chemicals to: evaluate Salmonella/microsome test (Ames Assay); evaluate newly developed bacterial tests; improve sensitivity of short term assays using mammalian organ enzymatic system preparation; and develop levels of test competence yielding most reproducible and accurate assays on most advantageous time/cost basis.

In Vivo Carcinogenesis. Conduct studies in vivo on a number of environmental chemical compounds potentially hazardous to man. Objectives are to perform studies in animals under strict holding and procedural requirements to assist in identifying carcinogenic chemicals based upon tumor development, and investigate causal relationship between pharmacokinetics of a chemical and its carcinogenic potential.

DCCP/FCRC Administrative and Scientific Support Area: Within the Contractor's DCCP/FCRC administrative area, there are several important ancillary staff, professional, and technical groups that provide necessary support for research operations and assist BCP and CCP Contractor directors in discharging their supervisory responsibilities.

The first of these is the administrative staff. This group, including various coordinative, clerical, and editorial personnel, furnishes assistance related to budgetary, personnel staffing, and facilities operations in the day-to-day handling of the Contractor's DCCP operations.

The second provides two important professional support functions involving high quality electron microscopy and quality control. The former furnishes necessary cooperative assistance for a variety of investigational, resource and collaborative research activities conducted within the total BCP program at FCRC. Particle counts, thin section preparations, and ultrastructural analyses are some general examples of the work performed by the electron microscopy staff. The Quality Control Group essentially provides routine testing services for the isolation and identification of mycoplasmas from cell cultures and serum in support of the BCP program at FCRC and for the Bureau of Biologics, FDA, via an interagency agreement. Pending availability of facilities and personnel, some testing may be performed for specific investigators outside of FCRC on a limited basis.

The third group of activities consists of technical support in the form of animal care, glassware preparation, and custodial services applied as required throughout the DCCP/FCRC.

Finally, as the chief Contractor authorities in the BCP and CGP areas, respective program directors have the responsibility for promulgating and maintaining adequate safety practices throughout the DCCP/FCRC Laboratory areas. This responsibility was shared on a designated basis with the various section

leaders to insure that an adequate safety awareness permeated all levels of research and support personnel activities. In pursuing this endeavor, close cooperation was established and maintained with FCRC Biohazards and Environmental Control. Their advice and assistance is secured in matters dealing with but not necessarily limited to: (i) establishing safe operational procedures to insure protection of the worker and experimental integrity, (ii) periodic facility and equipment inspections, (iii) facility designs for new or renovated areas, (iv) proper disposal of waste materials, and (v) adherence to prescribed safety regulations.

Other ancillary work which DCCP supports on a shared basis at FCRC is as follows:

Pathology and Histotechnology. To provide a uniform, flexible, and equitable service to all investigators at the Frederick Cancer Research Center requiring pathological and histotechnological procedures. Every effort is to be made to insure both excellent quality stained microslides in large quantities and a rapid turnaround period. In addition to the routine histological procedures performed on a daily basis, this group also assists in the performance of animal necropsies in cooperation with the Bioassay Research Program.

Environmental Control. To conduct a comprehensive safety and environmental control program for the Frederick Cancer Research Center and to perform applied and basic studies and literature surveys for risk assessment in support of the various FCRC operations.

Animal Breeding. To operate an animal farm for the breeding of laboratory animals to meet the needs of research programs at FCRC, and for shipment to other NCI operations as production permits.

Animal Health Diagnostic Section. To provide for monitoring the health and genetic quality of research animals at the FCRC and NCI in order to ensure the validity of animal-related research and to preclude the entry of undesirable pathogenic microbes, latent murine viruses and parasites.

Program Projections: As regards future DCCP/FCRC program development, DCCP/NCI may determine that particular research or resource goals require changes in emphasis; objectives may be altered and/or previously established ones postponed or eliminated. Conceptual modifications may be introduced, as for example the recent increased program interest in viruses and chemicals as cocarcinogens. Programs will be reviewed periodically by NCI and expert outside peer review groups and determinations made as to their need or desirability for continuation while new projects may be evaluated for potential program merit and exploratory effort. The continued need for alterations in the conduct of Contractor research will not connote expansion of the current budget. Quite the contrary, advantage will be taken of the fact that obsolete,

undesirable, or unproductive program effort(s) are to be phased out. These would be redirected into more fruitful research areas, for example, as described above, with the possible recruitment of outside scientific expertise, but without necessarily elevating personnel levels.

For the immediate future, the DCCP/FCRC Program, while directed toward attainment of essentially the same overall goals and objectives, will continue with important modifications of several of its component parts. In the Biological Carcinogenesis Program, the phase out of the Biology of Type C Viruses Section in the latter part of the current contract year was the result of its being judged as a relatively low priority effort. The same holds for the Endogenous Formation of Carcinogenesis Section in the Chemical Carcinogenesis Program. Moreover, the Endocrine Carcinogenesis Section was eliminated at the end of last year but may undergo some type of revival depending upon availability of suitable professional expertise. In the area of In Vivo Carcinogenesis, efforts with current chemical carcinogens will continue until final commitments have been achieved with respect to standardized testing of the compounds on behalf of the NTP. Facilities and resources will continue to be used, to a greater extent in support of mechanisms of nitrosamine carcinogenesis and, perhaps, other projects with critical animal holding requirements. A similar situation exists within the In Vitro Carcinogenesis Section. This effort will veer from work previously confined to development of assay procedures for chemical carcinogens and begin to probe into mechanisms of action related to chemical transformation of cells.

Both the Biological and Chemical Carcinogenesis Programs were designed to inherently provide strong but flexible scientific mechanisms that allow continued opportunity to enhance program productivity and effectiveness. Maximal use is made of results of previous efforts as a basis for more advanced work, for rapidly capitalizing on new scientific and technological advances, and for seizing new initiatives in previously untried areas of investigation as related to human cancer. As an illustration of this are DCCP's continuing plans to explore and foster where feasible genuine cross-collaboration of efforts between the Biological and Chemical Carcinogenesis Programs. Scientific cooperation among at least portions of these respective program endeavors should produce a unique blending of program interests and objectives, and provide significant infusions of scientific expertise. This will, in turn, undoubtedly open new research vistas for exploration, portending the attainment of program accomplishment of great importance in the elucidation of problems concerned with cause and prevention of human cancer.

Program Funding (Annual Level):

DCCP/FCRC (Full)*	$11,159.7
Other (Partial)**	1,071.6
ISA***	1,913.6
Total	$14,144.9

* Includes complete DCCP/FCRC funding for Biological and Chemical Carcinogenesis Programs, plus Contractor support for on-site NCI Carcinogenesis Intramural Programs. Also includes fixed and available award fees.

** Includes partial DCCP/FCRC budget support for Environmental Control and Animal Production Area

*** Represents DCCP portion of Interdepartmental Support Agreement for services furnished by U.S. Army.

LITTON BIONETICS, INC. (NO1-CO-75380), BETHESDA, MARYLAND

Title: Operation and Maintenance of the Frederick Cancer Research Center (FCRC), National Cancer Institute, Fort Detrick, Frederick, Maryland 21701

Contractor's Project Director: Dr. Michael G. Hanna

Project Officer (NCI): Dr. William W. Payne

Assistant Project Officer for DCCP/FCRC (NCI): Dr. Henry J. Hearn

Objectives:

A. Using all contemporary techniques of nucleic acid chemistry, determine role of retroviruses in normal and tumorigenic processes of the host species. Objectives are to: characterize viral genomes responsible for oncogenic properties; study molecular mechanisms of replication; map viral genomes to identify active fragments; determine relatedness among viruses and primate cell DNA; and develop cloning techniques to generate probes for screening human DNA.

B. Conduct immunological characterization and amino acid sequence analysis of retroviruses. Objectives are to study: structural similarities and phylogenetic relationships; role of viral polypeptides in assembly, maturation, transformation, and oncogenicity; cleavage of precursor polyproteins and processing enzymes; structure of immunologic determinants; synthesis of peptides corresponding to antigenic regions of virus proteins for use as immunologic probes and potential vaccines; analysis of virus-related proteins and development of immunochemical techniques for entire Biological Carcinogenesis Program.

C. Conduct investigation on mammary tumor viruses, which are unique in that they represent the only ones known to induce mammary carcinomas; are important models for solid tumors of epithelial origin. Objectives are to study: nature and control of expression of MMTV proteins, surface antigens, and other viral components; analysis of MMTV genomes for oncogenic potential; interaction of MMTVs with their hosts: cellular and humoral immune responses; comparisons of endogenous and exogenous MMTVs for immunology, genetic relatedness, tumor induction; and develop and use monoclonal probes for studies in human systems.

D. Develop primate models for studies on immunology and pathogenesis of lymphomas and leukemias. Objectives are to study: role of EBV-like Herpesvirus papio in spontaneous lymphoma in baboons; studies of herpesvirus isolates from chimpanzees, orangutans, and gorillas; pathogenesis and immunology of T-cell lymphomas induced by Herpesvirus saimiri in monkeys; and mechanisms of EBV-induced malignant transformation of cultured cells.

E. Investigate etiology and molecular events in induction of leukemias in murine models. Objectives are to study: T-cell differentiation and function

including T-cell antigen specificity; role of type C viruses and chronic immune stimulation in induction of T-cell leukemias; and comparative mechanisms of leukemia induction by radiation, hydrocarbons, urethan, and estrogens in mice.

F. To provide facilities and conduct a major resource effort for the large-scale production of viruses or viral components for use within the FCRC and for distribution through the NCI DCCP's Biological Carcinogenesis Branch Research Resources Program, by providing high quality retrovirus concentrates and conducting developmental research for eliciting and/or optimizing virus expression and recovery, and pursuing product improvement studies.

G. To provide high quality electron microscopic support for the DCCP/FCRC program in the form of particle counts and thin section screening of production cell lines; also to make visual determinations of viral RNA structure and apply immunoelectron microscopic methodology to studies on viral proteins.

H. To provide quality control assays for the detection of microbial contamination of all cell culture systems in use throughout the DCCP/FCRC program, particularly for the Viral Resources Laboratory.

I. Investigate carcinogenic potential of N-nitroso chemicals derived from environmental amino compounds and nitrite. Objectives include: mechanisms of formation of N-nitroso compounds; feeding of amines with nitrosating agents; investigate structure - neoplastic activity relationships; study potency and organ/species specificity of N-nitroso compounds; distribution of N-nitroso compounds and their precursors in the environment; and synthesis of N-nitroso compounds with known structure to determine metabolic pathways toward carcinogenesis (or detoxication) in various organs/species.

J. Elucidate mechanisms of carcinogenesis as related to chemical structure. Objectives are to: study how nitrogenous organic chemicals such as nitrosamines, triazines, hydrazines and related substances are activated to become carcinogens; suggest ways in which active intermediates interact with cellular components; and refine information on known or suspected biochemical mechanisms of carcinogen formation.

K. Elucidate molecular aspects of carcinogenic process related to interaction between carcinogen and/or its metabolites and host DNA. Objectives are to study reaction of alkylating agents with nucleic acid constituents to understand site selectivity; studies on properties of DMBA-deoxyribonucleoside excision; and compare different DMBA-DNA adducts and operative metabolic routes in vitro and in vivo and influence dose of carcinogen dose on these activities.

L. Elucidation of mechanisms in chemical carcinogenesis emphasizing transformation of epithelial cells. Objectives are to develop assays for in vitro transformation of liver epithelial cells (including human liver cells); investigate sequence of events in epithelial cell transformation; and identify the target cells for chemical carcinogens in liver.

M. Preparation and characterization of a variety of chemical carcinogens and development of analytical expertise to support DCCP/FCRC programs. Functions include efforts to: synthesize and purify carcinogens primarily in support of chemical carcinogenesis projects; develop and use analytical methods to characterize carcinogens and identify impurities; and develop new methods for synthesis and analysis of carcinogens.

N. Conduct electron microscopy studies to identify chemical reactions of the inducing chemical agent and characterizing the biological response of the affected cells. This includes work to: identify morphologically those cells that are targets for chemical carcinogens; identify morphological basis for early detection and diagnosis of precancerous lesions; and contribute to understanding mechanisms of action of nitrosamines and/or their precursors.

O. Development of short-term in vitro carcinogenesis bioassay systems for detecting carcinogens that may be hazardous to man and use these systems for studying mechanisms of chemical carcinogenesis, to provide information for preventing, inhibiting or reversing carcinogenic process. Objectives are to: study various metabolic activation, including enzyme, systems from various animal species that affect cell transformation; determine endpoints for activity such as elevation of aryl hydrocarbon hydroxylase and epoxide hydrase; study induction of metabolic activation enzymes in target cells in vitro or in donors in vivo; and examine cell cultural factors that enhance transformation response.

P. Provide a microbial mutagenesis testing service in conjunction with chemical carcinogenesis (and other) program elements for prescreening evaluation of mutagenic chemicals to: evaluate Salmonella/microsome test (Ames Assay); evaluate newly developed bacterial tests; improve sensitivity of short-term assays using mammalian organ enzymatic system preparations; and develop levels of test competence yielding most reproducible and accurate assays on most advantageous time/cost basis.

Q. Conduct studies in vivo on a number of environmental chemical compounds potentially hazardous to man. Objectives are to perform studies in animals under strict holding and procedural requirements to assist in identifying carcinogenic chemicals based upon tumor development, and investigate causal relationship between pharmacokinetics of a chemical and its carcinogenic potential.

R. To provide a uniform, flexible, and equitable service to all investigators at the Frederick Cancer Research Center requiring pathology and histotechnological procedures. Every effort is to be made to insure both excellent quality stained microslides in large quantities and a rapid turnaround period. In addition to the routine histological procedures performed on a daily basis, this group also assists in the performance of animal necropsies in cooperation with the Bioassay Research Program.

S. Recognizing that research activities at FCRC present potential safety problems in biological, chemical, radiological and industrial activities, a major effort will be organized and maintained to minimize hazards to FCRC personnel and to prevent the escape of potentially hazardous materials from the laboratory environment.

T. To operate an animal farm for the breeding of laboratory animals to meet the needs of research programs at FCRC, and for shipment to other NCI operations as production permits.

U. To provide an Animal Health Diagnostic Section for monitoring the health and genetic quality research animals at the FCRC and NCI in order to ensure the validity and animal-related research and to preclude the entry of undesirable pathogenic microbes, latent murine viruses and parasites.

In addition to the above contractor objectives are the following:

A. To furnish professional and technical support for a program in the Microbiology Section, Laboratory of Molecular Virology, to investigate mechanistic role of herpesviruses as potential carcinogens or cocarcinogens; to relate early type C virus activation in BALB/c cells treated with UV-irradiated herpesvirus and later occurring cell transformation; to map HSV genome, determine biologically active regions for thymidine kinase activity, type C virus activation, cell transformation; to characterize specific proteins in HSV-infected cells with respect to peptide maps and antigenic specificity; and to compare mechanisms of cell transformation by HSV with other DNA transforming viruses. Results of this work appear elsewhere in the annual report (Dr. Berge Hampar).

B. To furnish professional and technical support for a program in the Viral Genetics Section, Laboratory of Cellular and Molecular Biology, to conduct studies to gain understanding of the genesis of transforming viruses which may occur through a recombination between mammalian type C RNA helper viruses and host cellular genes, based on postulation that recombinant genes encode proteins that may be associated with malignant transformation; and also studies of nonprimate and primate retroviruses to characterize origin and function of translational products of mammalian transforming viruses; to explore possible role of type C viruses as vectors for cloning cellular genes with transforming function; and to determine whether structural and nonstructural proteins analogous to those encoded by type C transforming viruses are involved in spontaneous and chemically induced tumors. Results of this work appear elsewhere in the annual report (Dr. John Stephenson).

C. To furnish professional and technical support for a program in the Viral Immunology Section, Laboratory of Cellular and Molecular Biology, to develop, elucidate mechanism of action, and define effective biological conditions for vaccines for immunoprevention of spontaneous and/or chemically induced neoplasia in model systems; to study prevention of spontaneous and chemically

induced tumors by immunization with live viruses, transformed cell vaccines, and by passive immunization; to study cross-protective immunoprevention of transplantable rat tumors; and evaluation of tumorigenicity of virus and/or chemically transformed human and/or chimpanzee cells in nude mice or immunosuppressed rats. Results of this work appear elsewhere in the annual report (Dr. Robert Huebner).

D. To furnish professional and technical support for a program in the Viral Immunology Section, Laboratory of Cellular and Molecular Biology, to define naturally occurring cell-mediated immune responses to endogenous type C virus in the mouse; to characterize effector immune responses responsible for immunoprevention of endogenous type C virus expression and experimentally induced tumors; to determine whether naturally occurring humoral immunity to endogenous type C virus enhances or inhibits cell mediated immunity to type C virus; and to determine whether inactivated type C virus or virus containing cellular immunogens can prevent spontaneous or chemically induced tumors in mice possessing cellular and humoral immune responsiveness to the viral immunogens. Results of this work appear elsewhere in the annual report (Dr. Gary Kelloff).

E. To furnish professional and technical support for a program in the Biomolecular Oncology Section, Laboratory of Cellular and Molecular Biology, to study cellular recognition processes, to examine the influence of chemical and biological carcinogens on cellular differentiation and function and the application of model systems to elucidate biological, biochemical and immunological factors that modify host susceptibility to oncogenesis; and to develop an understanding of type C virus activation by, and interaction with, normal physiological, particularly immunoregulatory, processes. Results of this work appear elsewhere in the annual report (Dr. Arnold Fowler).

Major Findings:

Those project areas within the Contractor's FCRC operations that were completely funded by DCCP, NCI and for which major findings are given include the following:

Molecular Biology of Retroviruses. Retroviruses exhibit a complex biology containing, within the family, integrated noninfectious forms, horizontally-transmitted acute oncogenic forms, infectious weakly oncogenic members, and even genomes which exist in a viroid-like form. In order to determine the role of these viruses in the normal and pathobiology of the host species and potentially discover or create related members (or genes/gene products) in man, several models were selected for intensive study. The techniques employed in the work are common to the individual problems and include molecular cloning, restriction mapping using specific cDNA probes, DNA sequencing, heteroduplex mapping, in situ hybridization, in vitro translation of selected mRNA's, immunologic analysis of transforming gene products, and analysis of the function of these products by microinjection or other gene or product transfer techniques.

Acute transforming viruses have a readily understandable mode of generating oncogenic effects. These viruses carry a transforming gene which appears to represent a cellular gene captured by recombination with an otherwise benign retrovirus. How the product of this gene, which in the case of avian sarcoma virus resembles a protein kinase, effects transformation is not understood. Two acute transforming viruses each with unique attributes were studied. Reticuloendotheliosis virus, a horizontally-transmitted virus of avian species, appears (based on data) to be a recombinant of a helper virus derived from mammals (primates?) and a cellular sarc gene of birds. The target cell for transformation appears to be an early stage in the myeloid lineage. Objectives of the study are the isolation and characterization of the viral genomic region responsible for oncogenicity and the isolation of the presumptive protein product which results in cell transformation. The second transforming system under study is the rat sarcoma virus isolated by cocultivation of a helper virus producing cell and one transformed by a chemical carcinogen. The creation of the sarcoma virus may involve a retrovirus-like genome (similar to a viroid) which underwent recombination with the helper. This viroid may also have acquired a transforming sequence from cell DNA. The product of the rat virus has been identified thus making it possible to study expression in normal and tumor tissues in rats and other species including man.

In addition to the acute transforming viruses which specify a transformation-related product, work was also concerned with the nature of endogenous viruses in primates, systems in which multiple noninfectious virus occasionally emerge. The requirements for generation of an infectious virus are under study with viruses from baboons and rhesus monkeys. In the latter case, related sequences have been identified in higher primates and thus, by cloning methodology, it was hoped to isolate DNA fragments which could be used to generate new viruses. This project has the long-term goal of detection of viral related sequences in man. If such exist, the use of chimpanzee or gorilla sequences as probes may detect them where rhesus-derived probes may not because of the extent of evolutionary divergence. This Section has also included a project on development and validation of a biochemical procedure for prophage induction as an assay for carcinogens and anti-tumor agents.

Immunochemistry. This Section is concerned with the immunochemical characterization and amino acid sequence analysis of retrovirus proteins. Such studies necessarily involve a large effort for obtaining chemically and antigenically homogeneous purified proteins using conventional techniques. However, the development and adaptation of novel methods for protein purification, immunochemistry, and microsequence analysis, including sequencing by intrinsic radioactivity, represent another major effort of this Section. Several major developments in these areas have been detailed, for example: The NH_2-terminal sequences (of substantial lengths) of the α and β chains of avian myeloblastosis virus (AMV) reverse transcriptase were determined using amounts of proteins in the hundred picomole range. Degradation of subnanomole amounts of feline leukemia virus (FeLV) gp70 also permitted the determination of its NH_2-terminal sequence (18-20 residues). A significant sequence homology with

Rauscher leukemia virus (R-MuLV) gp70 was found, which involves a 14 residue segment around a carbohydrate attachment site. By using either in vivo or in vitro synthesized proteins which were metabolically labeled with radioactive amino acids, partial amino acid sequences were determined for avian sarcoma virus (ASV) pp60^{src} and R-MuLV *env* gene encoded precursor proteins Pr68^{env} and gPr85^{env}. Other major findings included are: The elucidation of the complete primary structure of R-MuLV p10 and the almost complete sequence identities of all mouse type C virus nucleoproteins. The nearly complete amino acid sequence of R-MuLV p30. The correlation of amino acid sequences with nucleotide sequences available in the literature. The new classification of type C viruses based on NH_2-terminal sequences of p30s. The development of novel methods for the purification and characterization of retrovirus phosphoproteins and nucleic acid binding proteins. The demonstration of type-specific antigenic determinants of p30s and gp70s as being primarily conformational sites and the group and interspecies determinants as being primarily sequential (continuous) sites. The partial antigenic mapping of R-MuLV p30. The delineation of the primary structure of an interspecies shared antigenic determinant of p30s by using synthetic peptides and hyperimmune sera and/or monoclonal antibodies. The isolation of two genetic variants of R-MuLV and partial characterization of their *env* gene products.

The Immunochemistry Section is also responsible for the analysis (composition, sequence) of viral and viral-related cellular proteins for the entire Biological Carcinogenesis Program. Most recently, a central sequencing facility was established within the Section in order to carry out protein sequencing studies for other Program areas at the Frederick Cancer Research Center.

Molecular Biology of Carcinogenesis. This Section is concerned with: (i) the control of expression of MMTV components (proteins and nucleic acids); (ii) the distribution of MMTV cell surface antigens as potential immune targets; (iii) the role of cellular and humoral immune responses to MMTV and their relationship to the development of spontaneous mammary tumors (both viral and chemically induced); (iv) the mechanism of hormone action; (v) the analysis of the MMTV genome by cloning and nucleotide sequencing to determine which viral genetic sequences are involved in oncogenicity; and (vi) the development of monoclonal antibody probes to differentiation and tumor-specific antigens.

This Group has already made considerable progress in elucidating the MMTV gene order and the steps leading to the synthesis and assembly of a viral particle. This includes the steps involved in viral membrane biogenesis. Based on studies of natural immunity together with the development of monoclonal antibodies to MMTV, we have defined in detail the antigenic polymorphism of the MMTV major glycoprotein and identified those determinants involved in viral infectivity (viral cell surface receptor binding). These natural mouse sera studies have shown that the mouse develops three functionally-distinct antibody populations, (i) class-specific neutralizing antibodies, (ii) class-specific cytotoxic antibodies, and (iii) group-specific precipitating antibodies. In addition, epitopes with group-, type-, and class-specificity have been defined with the monoclonal antibodies.

To gain further understanding of the oncogenic potential of MMTV, cell lines have been developed which produce either the exogenous or the endogenous forms of MMTV. Results indicate that the exogenous MMTV is highly oncogenic whereas the endogenous MMTV exhibits a much lower degree of oncogenicity. The comparison of the genomes of these two viruses has provided an ideal model for sequence analysis of clones MMTV DNA and uncloned MMTV RNA in determining those sequence differences which correlate with oncogenicity.

Primate Virus Immunobiology. Essentially, the projects currently under study all extend from an initial intent to develop primate models for the study of the immunology and pathogenesis of lymphomas and leukemias. At the time the first studies were begun there was only one experimental model available. This was the experimental induction of lymphoma and leukemia in marmosets and owl monkeys with Herpesvirus saimiri. The work of several laboratories around the world has made this system the most extensively studied primate tumor system currently under investigation. The differential responses of marmosets, owl monkeys, and squirrel monkeys to this virus, the varied immune responses to the virus, and the analysis of target cells continue to make the H. saimiri-lymphoma the most interesting model of primate T cell disease available for study. Work on H. saimiri oncogenesis led to a collaboration with Professor Anthony Epstein on similar investigations of the oncogenic potential of the Epstein-Barr virus. The first evidence for tumor induction by EBV was obtained and eventually it was first shown that the experimental B cell disease which it induces is monoclonal as is Burkitt's lymphoma. The work with EBV in turn led to a continuing collaborative program with Professor Boris Lapin of Sukhumi, U.S.S.R. Baboons in the large research colony at Sukhumi have a high frequency of spontaneous lymphoma. Research resulted in the isolation of an EBV-like virus of baboons, H. papio, and subsequent research has provided evidence that this virus is related to baboon lymphoma in the same fashion that EBV is related to Burkitt's lymphoma in man. Soon after the isolation of H. papio, similar viruses were isolated from chimpanzees, orangutans, and gorillas, the latter two isolates from this laboratory. It thus became apparent that EBV was a member of a large group of B cell tropic herpesviruses of Old World primates. There then began a program of more precisely defining the relationships of these viruses to each other and to EBV.

These studies, to date, have been concerned with comparisons of the antigenicity, nucleic acid structures, and biological activity of these viruses. The laboratory is now doing detailed investigations in immunology and pathogenesis, virology, and cell biology. Interests were extended into the mechanism of EBV transformation. There is now a pilot study to see if cells from patients with a genetically determined predisposition to cancer would behave differently after transformation with EBV than cells of normal individuals. In effect, this is a project to determine if EBV transformation actually represents tumor cell promotion.

In a similar natural progression studies were begun on production of monoclonal antibody by the murine hybridoma procedures as a means of raising precise immunological reagents to viral and cellular antigens. Initial efforts in this direction were made with Herpes simplex virus because this provided a system on which to learn, which dealt with herpesviruses and which was technically ripe for such studies. The production of type-specific antisera to HSV-1 and HSV-2, which are potentially available for epidemiologic studies in man, validated this judgement. In a similar fashion, there is now the need to expand understanding of the basic immunochemistry of primate lymphoid tumor cells. Thus far an extensive array of unique cell lines from a variety of primate species have been developed.

Mechanisms of T-Cell Leukemogenesis. This Section is concerned primarily with the etiology of and molecular events operative in the induction of leukemias in experimental models. Emphasis has been on radiation-induced leukemias in C57BL/6 mice, the spontaneous leukemias of AKR mice, and Moloney leukemia virus (MoLV)-induced leukemias in BALB/c mice. By necessity, the approaches used have involved studying a variety of fundamental aspects of viral immunity, viral genetics, T-cell differentiation, and T-cell function. Several major advances have been made in these areas allowing a unique integrated approach to elucidation of the mechanisms involved in virus-induced leukemogenesis.

In the area of T-cell differentiation, specific enzyme markers for two unique subpopulations of T cells have been developed. Moreover, it has been demonstrated that certain viral leukemias can involve either population. This information is currently being used to determine the possible relationships between these populations, the factors which control their differentiation and immunological functions, and their contributions to the leukemogenic process.

Experimental models have also been developed to study biochemically various aspects of T-cell antigen recognition and function. There have, or will be used, purified viral proteins, monoclonal antibodies, and anti-idiotypic reagents in these studies. The goal is to identify antigen-specific T-cell factors and T-cell antigen receptors to further understand T-cell functions. These experiments are coordinated closely with the above studies on T-cell differentiation and together, will contribute to a better understanding of T-cell function, and hopefully, leukemogenesis.

Studies of the immune responses against type C viruses in preleukemic mice have revealed the unexpected result that chronic T-cell stimulation may contribute to leukemogenesis. This result has led to the development of specific methods for establishing T-cell lines in vitro and, possibly, for establishing in vitro techniques to study viral leukemogenesis. Efforts will be directed to full characterization of this process with regard to virological and immunological specificity.

Biology of Type C Viruses. Prior to the phase-out of this effort early in 1980, the broad purpose of this Section was to understand factors involved in regulating gene expression. Chemical activation of latent type C viruses was chosen as a model system for study. In the past year, two key events in the induction process have been identified, and chemical ways of blocking the induction have been found that may lead to the identification of other steps and the provision of a means for controlling expression. By analogy, with induction of lambda phage from bacteria, proteolysis seems to be required for virus induction to occur. Two distinct classes of protease inhibitors, the trypsin inhibitor, α-N-tosyl-L-lysine chloromethyl ketone (TLCK), and two naturally occurring oligopeptide inhibitors, antipain and leupeptin, were found to block induction when present before and during, but not after, chemical activation. Since viral specific RNA has been shown to accumulate during type C virus induction, a direct test for transcriptional derepression involving a DNA-dependent RNA polymerase was undertaken. A mouse cell system which retains inducibility of type C virus following reversible permeabilization by salt was used to test the sensitivity of the induction process to α-amanitin. The sensitivity of the cellular type C virus induction process to variable concentrations of α-amanitin and the in vitro sensitivities of partially purified polymerases I, II, and III from the same cells indicate that an enzyme activity resembling host cell RNA polymerase II is involved in transcription and activation of the type C virus genome.

Due to program-wide interest in defined retroviral proteins, this Group undertook the task of isolating and characterizing low molecular weight viral DNA binding proteins. At present, 10 such proteins were purified to homogeneity and characterized by amino acid composition, molecular weight, and isoelectric point by two-dimensional gel electrophoresis.

Scientific Support Area. The Electron Microscopy Group develops and applies the techniques of scanning (SEM) and transmission (TEM) electron microscopy to meet the needs of investigators in the Biological Carcinogenesis Program on both a collaborative and a support basis. Although classical techniques for ultrastructural morphologic studies of viruses and cells are still frequently required, recent emphasis has been placed on the molecular aspects of electron microscopy (EM), including immunoelectron microscopy (IEM) and nucleic acid size, structure, and homology analysis by modification of the Kleinschmidt technique and heteroduplex mapping.

The specific objectives of the Electron Microscopy Group are to: (i) develop IEM markers and techniques and perform studies on the localization of viral proteins in the electron microscope; (ii) perform studies on the size, structure, and relatedness of retrovirus genomes; (iii) provide a quality control service to the Viral Resources Laboratory; and (iv) support collaborative efforts of the Biological Carcinogenesis and NCI Carcinogenesis Intramural Programs.

The primary objective of the Quality Control Group is to provide routine test services for the isolation and identification of mycoplasma from various sources including cell culture and serum.

The following procedures are currently in use for routine mycoplasma testing. Cell culture specimens are incoulated into agar medium and the outgrowth of mycoplasma colonies is detected by microscopic observation. For identification, unfixed mycoplasma colonies are reacted directly with fluorescein-conjugated globulin and examined by use of incident UV excitation. Additionally, indicator cell cultures are used to detect mycoplasma contaminants in cells. Test specimens are inoculated into 3T6 cells growing on coverslips. After incubation, one of the two coverslips is stained with a fluorescent antibody conjugate directed against M. hyorhinis. The second coverslip is stained with a fluorescent chromatin stain. All mycoplasmas which have been reported as contaminants in cell cultures can be detected by this combination of procedures.

The use of a prescribed cell culture system for research is predicted on the absence of unprescribed organisms. The rationale of the Quality Control Group is to develop and utilize the techniques required to support the Biological Carcinogenesis and Carcinogenesis Intramural Programs in mycoplasma control.

Viral Resources. The Viral Resources Laboratory (VRL) consists of three highly integrated groups - Virus Production, Virus Purification, and Developmental Biology. This effort serves both the NCI Division of Cancer Cause and Prevention's Biological Carcinogenesis Branch (DCCP/BCB) and the Center's Biological Carcinogenesis (BCP) and Carcinogenesis Intramural (CIP) Programs in providing well-characterized retrovirus concentrates and related products, in conducting developmental research aimed at eliciting and/or optimizing virus expression and recovery, and in providing further technical support to BCP and CIP investigators. Throughout contract year 1979 (February to September 1979), VRL commitments entailed the production, purification, and characterization of 600 liters per week of retrovirus material, essentially equally distributed through DCCP/BCB and BCP. In contract year 1980 (October 1979 to present), production commitments were reduced to a total of 450 liters per week as follows: DCCP/BCB - 240 liters/wk; BCP - 180 liters/wk; and CIP - 30 liters/wk. Viruses produced for DCCP/BCB were few in number, consisting mainly of Rauscher murine leukemia virus (R-MuLV) and mouse mammary tumor virus (MMTV), principally from C3H murine cells, but also including MMTVs of RIII and GR murine origin. In contrast, material for BCP and CIP investigators was generated from over 60 different producer cell lines. In 1979 (February 1979 to January 1980), 25,016 liters of retrovirus-containing cell culture fluids were produced within VRL. Included in this volume were: 11,997 liters (48%) C3H-MMTV, 2,471 liters (9.9%) R-MuLV, and 10,548 liters (42.1%) from 60 other cell lines. The Laboratory thus maintained its high capacity, high diversity production record in 1979; in the more than seven years of its existence, VRL has produced 160,527 liters of virus material from 109 different cell lines. Distribution of 24,989 milliliters of virus concentrate (average concentration, 956X) was accomplished in 1979, resulting

in the fulfillment of all contractural commitments. Distribution through DCCP/BCB totaled 12,505 milliliters. The FCRC Biological Carcinogenesis Program received 11,015 milliliters in 1979; CIP investigators received 483 milliliters. Internal characterization required 986 milliliters or 3.9% of total distribution. Since 1972, VRL has distributed 166,990 milliliters (average concentration, 96%) of material.

Mechanisms of Nitrosamine Carcinogenesis. This Section is devoted to the study of a variety of aspects of nitrosamine carcinogenesis including the possible involvement of human exposure to carcinogenic nitrosamines in human cancer. Studies related to environmental exposure to nitrosamines include the mechanism of nitrosation of tertiary and secondary amines, transnitrosation of amines by aliphatic nitrosamines, and the isolation and identification of secondary and tertiary amines in the environment. Assessment of the significance of some of these reactions in vivo is attempted by chronic feeding of several important amines together with nitrite or with a nitrosating N-nitroso compound to rats. Tumor induction in the animals is an indicator of the formation of a carcinogenic nitrosamine in the body.

Many N-nitroso compounds of different structures have been synthesized, often by novel routes developed here, for administration to rats, hamsters, or mice with the objective of understanding the relationship between chemical structure and carcinogenic activity among this group of compounds. Oral administration to rats of nitrosamines and skin painting on mice of nitrosamides are used. The carcinogenic effectiveness of the N-nitroso compounds is compared with their mutagenic effect in Salmonella typhimurium strains.

Using the carcinogenicity data as a base, the metabolism and activation of several nitrosamines are compared in target and nontarget organs of rats, hamsters and, in some cases, guinea pigs, as well as in the whole animals. The objective is to determine those reactions related to carcinogenesis.

Chemistry of Carcinogens. The past year has seen considerable progress in research into the development of the concepts which were presented in the 1979 report. The study of the in vitro metabolism of ^{15}N-labeled nitrosamines has shown that dimethylnitrosamine is oxidized almost exclusively by the α-hydroxylation pathway. This suggests that the methyldiazonium ion is the ultimate carcinogen from this nitrosamine. The situation with phenylmethylnitrosamine is more complicated, but again, the oxidation of the methyl group seems to be the principal pathway of metabolism. Studies are now continuing with dibutylnitrosamine. Studies of the in vitro metabolism of phenylmethylnitrosamine, using a combination of enzyme kinetics and bacterial mutagenesis data, have indicated that it will now be possible to separate the enzymatic activation sequence from the reaction sequences responsible for mutagenesis.

The first general preparation of trialkyltriazenes has resulted in a potentially very useful tool for the study of the in vivo effects of the alkyldiazonium ion. This is due to the fact that the triazenes decompose readily

to an alkyldiazonium ion without enzymatic intervention. Consistent with that, all the trialkyltriazenes studies have been potent mutagens in the Ames assay. The chemistry of O-alkylated nitrosamines (alkoxydiazenium ions) has provided another possible twist to the already very complex chemistry of the N-nitroso function. Thus, the reaction of these materials with nucleophiles is best explained by involving an initial intramolecular rearrangement of the O-alkyl group to the amino nitrogen. N-nitrosoenamines have proven to have a very rich chemistry, as was suggested in the 1979 report. There is reasonable evidence that some of these substances may be found in the environment, and others may be formed in vivo. Thus, it was found the Δ^3-N-nitrosopiperidine is isomerized to the Δ^2-isomer in vivo in the rat. Since the reverse transformation does not occur and since both isomers give exactly the same spectrum of tumors in the rat, it is reasonable to suggest that the N-nitrosoenamine (i.e., the Δ^2-isomer) is the substance responsible for the tumorigenic activity.

Molecular Aspects of Chemical Carcinogenesis. While it has been accepted for a decade now that the chemical reactivity of a carcinogen or its metabolite is the key common feature among chemical carcinogens, little progress has been made in defining more precisely the kind of chemical reactivity which is associated with carcinogenic potency. Studies on the reactions of aralkylating agents with nucleic acid constituents offer promise for real progress in this area. These studies describe for the first time a rational explanation for the selective reaction of different carcinogenic chemicals on various receptor sites on guanine residues. Thus, in order to modify either the O^6- or N^2-sites in guanine residues, instead of or in addition to the N-7 site, a reactive substrate needs to exhibit some degree of S_N1 character in its reactions. Furthermore, if the substrate tends to localize charge at the reaction center, exocyclic substitution will occur on the O^6-site, e.g., alkylating agents and alkylnitroso compounds. However, if the substrate tends to delocalize charge on the reaction center, e.g., aralkylating agents, hydrocarbon diol-epoxides, etc., exocyclic substitution will occur on the N^2-site. In the case of benzylating agents which delocalize charge but not as extensively as polycyclic aromatics, exocyclic substitution on both of these sites can and does occur. This novel understanding of the basis of site selectivity in nucleoside alkylation not only improves insight for predicting new substrates but allows work to begin to characterize chemical reactivity in such a way that certain types of reactivity specifically associated with carcinogenic potency might be recognized.

While research into the fundamental chemistry of carcinogen-nucleic acid interactions has been in progress, study of the chromatographic and fluorescence properties of DMBA-DNA adducts has allowed the demonstration that this most potent of the hydrocarbon carcinogens is activated through the bay region diol-epoxide mechanism. Also, certain peculiarities in the fluorescence of DMBA-deoxyribonucleoside adducts have been noted; and it has been found that these adducts are very slowly excised (in comparison with those formed by 3-mtheylcholanthrene) in embryo cell cultures from mice sensitive

to this carcinogen. These observations have generated the working hypothesis that hydrocarbons with a methyl substituent in the bay region (DMBA, 5-methylchrysene, 11-methylbenzo[a]pyrene) may owe their carcinogenic properties in part to an unusual conformation of hydrocarbon-deoxyribonucleoside adducts leading to slow excision of these adducts. The comparison of DMBA-DNA adducts formed in intact cellular systems, including mouse skin, with those formed in liver microsomal or S9 preparations has indicated that these latter systems may have a limited usefulness in conjunction with short-term test systems for chemical carcinogens. They generate a wider variety of DMBA-DNA adducts than do intact cellular systems and the involvement of the various metabolic routes operative in the homogenates varies with carcinogen dose. Thus, even dose-response relationships in such short-term tests do not necessarily indicate confidence with respect to the potential carcinogenicity of the test compound.

Cellular Aspects of Carcinogenesis. Research in this Section is focused on those stages of the carcinogenic process wherein epithelial cells acquire a malignant potential and escape the proliferation controls exerted by neighboring normal cells.

During this report period, objective criteria for epithelial cell transformation and experimental protocols for the selection of transformed cells have been developed. Investigations of the control of cell proliferation by endogenous factors and studies on the biochemical properties and responses to hormones of normal liver cells and hepatoma cells also were undertaken. In addition, there was established a large number of liver epithelial cell lines from different strains of rats and from regenerating rat liver (after partial hepatectomy), all of which will be used as the material for the proposed carcinogenesis experiments. Moreover, long-term autoradiographic studies in mouse skin, aimed at recognizing the putative initiated cells and examining their persistence in vivo, have been started.

Minor reorganization and recruitment within this Section resulted in enhanced focus of several areas in in vitro and in vivo studies of carcinogenesis. A cell-hybrid model has been established in this laboratory by fusion of rat liver epithelial cells with hamster ovary cells. This cell-hybrid model is being used as a positive control in experiments on carcinogenesis in vitro and as a model for screening carcinogens.

Chemical Synthesis and Analysis. This Section provides expertise, new technologies, methods development and support services in synthetic and analytical chemistry to the Chemical Carcinogenesis Program and the Frederick Cancer Research Center. The Section has initiated collaborative research projects centered around the synthesis and analysis of labeled (^{2}H, ^{3}H, ^{14}C) and nonlabeled carcinogens, their suspected metabolites, bioassay chemicals, and other chemicals important to cancer research.

The preparation and purification of 53 labeled (^{2}H, ^{3}H, ^{14}C) and nonlabeled chemicals, not readily available from outside sources, have been carried out

for various projects in the Chemical Carcinogenesis Program during the past year. The use of the diene-quinone reaction for the synthesis of PAHs has continued. This reaction was found to be extremely useful for the preparation of potential DMBA and 2-aminoanthracene metabolites. The advantages of the method are the relatively high yields and considerable savings in effort, as compared with alternative methods of synthesis.

New analytical methods were developed and some literature methods were modified for the separation, detection and quantitation of labeled and nonlabeled carcinogens, their suspected metabolites, bioassay chemicals, and other chemicals important to cancer research. Improved gas-liquid chromatographic separation of bile acids, steroids, methoxyquinones, and pesticides were developed using nematic liquid crystal phases. These separations represent significant improvements over those found in the literature. Labeled biosynthesized bile acids, benzo[a]pyrene and 2-aminoanthracene metabolites were analyzed by new methods developed for high pressure liquid chromatography. Methods were also developed for the analyses of urine metabolites of diethylnitrosomethylurea, nitrosomethylaniline and 2-acetylaminofluorene. Cryogenic, triangular, and two-phase TLC methods were developed for the separations of nitrosamines, steroids and other metabolites. The development of a sensitive and specific TLC spray reagent for the differentiation of amines (1°, 2°, 3°), nitrosamines, nitrite and nitrate was accomplished. The addition of a high resolution mass spectrometer has greatly increased our capabilities for structure elucidations and identifications of small amounts of unknown compounds for collaborative studies within the Chemical Carcinogenesis Program, the Biological Markers laboratory and the Fermentation laboratory. Last year 531 samples were submitted for MS (EI/CI/FD modes of ionization) analyses and 818 samples were submitted for ^{1}H and ^{13}C NMR analyses. Proton and ^{13}C NMR analyses were used for the identification of unknown chemicals and metabolites. The use of molecular spectroscopy UV-Vis and infrared have also increased over the past year.

The diene-quinone reactions for the synthesis of PAHs and their potential metabolites and the development of liquid crystal GLC phases for the separation of complex mixtures were directly applicable to long-term problems in Chemical Carcinogenesis.

Ultrastructural Studies. The work of this group focuses on three major objectives: (i) early diagnosis of precancerous lesions, (ii) identification of target cells, and (iii) organotropy of nitrosamines. Most of the experiments concentrate on pulmonary and pancreas carcinogenesis, while cancer in other organs (e.g., liver, kidneys) is being examined in collaborative studies. The methods used are very similar in all these experiments. The development of chemically induced tumors is studied sequentially by light and electron microscopy in serial sacrifice studies using established animal models. From these experiments, precancerous lesions and the cells from which the tumor originates (target cells) can be recognized. Parallel light and electron microscopic autoradiographic studies with labeled carcinogens help further in the identification of the site of binding (and most probably metabolism). To

provide a basis for extrapolation of the findings to man, comparative studies are carried out in vitro using both animal and human tissues.

As a result of work during the past year, the pancreatic acinar cells as the main targets of N-nitroso-2,6-dimethylmorpholine in Syrian hamsters have been identified; also identified basal cells as the source of diethylnitrosamine-induced tumors in the Syrian hamster trachea; and demonstrated that DEN and/or its metabolites cross the placenta in Syrian hamsters and bind to the fetal target organ (trachea) only during the last four days of gestation (during which time DEN is carcinogenic to the progeny). In collaborative studies, we have clarified the pathogenesis of kidney tumors induced in rats by the flame retardant Tris(2,3-dibromopropyl)phosphate), and have characterized a number of spontaneous lesions in aging Fischer rats.

In Vitro Carcinogenesis. Previous efforts in this Section have resulted in the standardization of procedures for using Syrian golden hamster embryo cells in an in vitro carcinogenesis bioassay by employing pretested aliquot samples of primary cultures cryopreserved in liquid nitrogen as the source of target and X-irradiated feeder cells. When over 121 reference chemicals were tested, results generally concurred (93%) with those observed in animal bioassay systems. Transformation was observed with most carcinogens and no false positives were observed when noncarcinogens were tested. When liver homogenate S9 preparations or cultured hepatocytes were added to several carcinogens apparently requiring further metabolic activation, all induced transformation. Further evaluation of the system was continued by testing a number of chemicals under double-blind conditions. In this contract year, major emphasis was placed on increasing the sensitivity and transformation response of the bioassay system to eliminate false negative results and to detect very weak carcinogens. Studies were concerned with: (i) The incorporation of metabolic activation systems, such as cultured hepatocytes or liver enzyme preparations, into the routine use of the standard bioassay. In collaborative studies these activation systems were further evaluated in the Salmonella mutagenesis system to determine the range of diverse carcinogens that can be optimally activated to mutagenic forms. (ii) The identification and selection of highly responsive target cells, which vary among batches, by using biochemical and enzymatic markers to predict usefulness. (iii) The in situ induction of activating enzymes in cultured target cells by using selected inducers. (iv) The optimization of culture conditions for chemically induced transformation by the addition of bacterially conditioned media or other factors.

Microbial Mutagenesis Screening. The Salmonella/mammalian-microsome mutagenicity test and the Escherichia coli repair test were used to screen 424 chemicals for their ability to induce DNA damage. The use of these two in vitro assays greatly increases the likelihood of detecting potentially carcinogenic compounds.

The use of microsomal preparations from different species of animals induced with various enzyme stimulators can increase the sensitivity of these tests.

A BioTran II Automatic Colony Counter greatly reduces the amount of time spent in counting revertant colonies. With the system properly programmed, there is an increase in accuracy with a decrease in technician fatigue.

A new method for the detection of mutagenic metabolites has been devised to rapidly screen human urine. This technique is being used in a study to determine if personnel associated with the oncological units of the National Cancer Institute are assimilating chemotherapeutic drugs.

In Vivo Carcinogenesis. Twenty-four month bioassays were conducted with Fischer 344 rats and $B6C3F_1$ mice. The protocols for these bioassays are developed according to NCI Guidelines for Carcinogen Testing in Small Rodents. Animals are received from the Mammalian Genetics and Animal Production Section of NCI, and a maximum tolerated dose (MTD) is determined for each test chemicals by preliminary toxicity tests. After each MTD is determined, animals are orally (feed, drinking water, or gavage) dosed with each chemical at the MTD and 1/2-MTD level for 24 months. At this time any surviving animals are killed. A full necropsy, as outlined in NCI Guidelines for Carcinogen Testing in Small Rodents, is performed on all test animals. Feed and water consumption, body weights, and clinical signs are followed throughout the course of each bioassay. The Chemical Synthesis and Analysis Section determines the purity and chemical stability of the test substance in the vehicle (i.e., feed/water) before each bioassay.

Eight chemicals are currently in one stage or another of bioassay testing. These chemicals are: Telone, Mirex, Randox, cyclohexanone, styrene oxide, benzyl chloride, methapyrilene, and nitrosodiethanolamine. All of these chemicals were from the list approved by the Chemical Selection Working Group of NCI and had the additional characteristics of relevance to one or more areas of work in the Chemical Carcinogenesis Program at FCRC, or were of particular interest for broader reasons (as in the case of Mirex).

Animals from the chronic bioassay of both Telone and Mirex have already been dosed for 24 months and have been killed on schedule. Results from the Telone study are being evaluated statistically and the histopathology of animal tissue from the Mirex study is currently being evaluated.

The chronic bioassay of cyclohexanone was started in April 1979 and is progressing satisfactorily.

Dose-finding studies with styrene oxide, benzyl chloride and methapyrilene were completed in early 1979. Data from these toxicity studies were sufficient to determine MTD in both rats and mice for all three chemicals. More significantly, methapyrilene was shown to be carcinogenic for rats in the subchronic test, thus alleviating the expense and time of conducting a chronic bioassay. The recommended MTDs for benzyl chloride and styrene oxide have already been presented to and accepted by NCI. Chronic bioassays of both chemicals were started in late 1979 and will continue for two years.

A dose-finding study with nitrosodiethanolamine was started in August 1979 and the animals killed on schedule in February 1980. Preliminary data have already suggested a MTD within the range of the subchronic dose levels; therefore, the determination of a MTD for the chronic bioassay should be straightforward.

Additionally, pharmacokinetic studies with bioassay test chemicals are being developed. These include gut absorption, organ distribution, metabolism, and metabolic balance studies with the test chemicals. The studies are designed to help in explaining dose-response results, target organ phenomenon and, perhaps, the relationship between chemical structure and carcinogenesis.

Those project areas within the Contractor's FCRC operations that were partially funded by DCCP, NCI on a shared basis, and for which major findings are given include the following:

Environmental Control and Research. The Environmental Control Section's objective is to provide a safe and healthful workplace for all employees. To realize this objective, a program has been instituted that encompasses the following areas of safety control: the biological safety program covers all laboratories and experimental animal holding areas where oncogenic viruses are under investigation and other potentially hazardous biological procedures, such as recombinant DNA studies, are carried out; the chemical safety program covers all laboratories and experimental animal holding areas where potentially hazardous chemicals, including chemical carcinogens, are used; a radiation safety program is maintained in all laboratories where radioisotopes or radiation-emitting equipment is used; and the industrial safety program implements physical safety and fire prevention techniques in all areas of the FCRC. The Environmental Control Section utilizes information from the environmental research, medical services, and safety information programs as well as from sources outside the FCRC to ensure that the safety program is responsive to the needs of the community it serves.

The responsibility of the Medical Services Group is to develop and implement laboratory, clinical, and epidemiological medical surveillance techniques that will reveal potential health and environmental problems. Full understanding of occupational illnesses and injuries and the proper care of such conditions are important aspects of the program. Emphasis is on prevention; however, when this is not possible, an effort is made to eliminate or minimize short- and/or long-term effects of these conditions. The development of a data compilation system for medical and exposure records and revision of medical forms are planned. This allows the Group to analyze the multiphasic health test, to evaluate exposures, to correlate abnormal tests, to identify specific work groups that will require periodic assessment, and to identify employees with disease disabilities that could be aggravated by continued exposures. The Medical Services Group utilizes information from the environmental control, environmental research, and safety information programs as well as from sources outside the FCRC to ensure that employees receive the kind of medical services they require.

The objectives of the Environmental Research Section's program are identified by considering the potentially exposed individual, the substances to which he may be exposed, the environment in which exposure may take place, and the interactions among these. To realize the objectives, guidelines for safe

practice are prepared and research is carried out to assess the risk associated with various common laboratory procedures, to determine the efficiency of adsorbents for the removal of non-particulate contaminants from air, to test the effectiveness of protective equipment, and to arrive at techniques for the degradation and disposal of chemical carcinogens. The Environmental Research Section utilizes information from the environmental control, medical services, and safety information programs as well as from sources outside the FCRC to assure the responsiveness of the research program to the needs of the scientific community.

Central Histotechnology Laboratory. During the past year a total of 334 animal necropsies were performed on Bioassay animals and 35 necropsy examinations were performed on animals from Dr. Lijinsky's studies, plus the tissue trimming of 3,700 animals from these studies. Also, during this period the Central Histotechnology Laboratory completed and forwarded to the pathologists a total of 17,640 stained microslides generated by the Bioassay studies and 44,970 stained microslides were prepared and submitted to FCRC investigators from other research programs. During the coming months, the laboratory will assist in the performance of approximately 550 animal necropsies in addition to its slide preparation.

Animal Resources. In the area of disease prevention and control, FCRC facilities are densely populated with animals that have been born, raised, and held under protected conditions. They have had no contact with disease organisms and, thus, have no immunity to the common diseases of rodents. This combination of dense populations and immunologically naive animals is a time bomb.

The first line of defense is to assure that any animals added to the populations are free of disease. The second line is the protective facility and its operating methods that protect against other sources of infection. But, no functioning animal facility can be absolutely protected. Therefore, a method for quickly detecting infections before they spread through the facilities is necessary. The Animal Health Diagnostic Laboratory provides the technical support for a program of monitoring for microbes in the animal facilities and for diagnosis of disease.

Services to receive and quarantine animals that must be obtained from outside sources are provided by an in-house group. The value of the quarantine and testing of incoming animals was demonstrated again this year when about 10% of the 253 shipments of animals received were rejected for reasons of health. Unchallenged acceptance of these animals would have produced serious health effects in the experimental animals.

In the animal production area, animal production during the year showed significant gains. Animal production matched to internal and external demands for disease-free animals and improved the efficiency of production. Facilities were modernized to eliminate physical obstructions to the achievement of quality. Extensive microbiological evaluation objectively demonstrated that the animals remained free of organisms causing epizootic rodent diseases. This year an additional four strains of mice and one of rat have been raised

to the barrier level of quality and, as they expand, will replace their counterpart colonies at the "interim" level of quality.

Gnotobiotic operations have overcome many detailed and technical difficulties in the extremely demanding procedure for aseptically obtaining full-term fetuses, transferring them to a sterile environment, reassociating them with selected microbes, nurturing them, and inducing them to multiply in such a controlled atmosphere.

Barrier Production continues to achieve new levels of efficiency and quality. This is adequately illustrated by a single outstanding example: The nude mouse, which is a notoriously poor breeder and is extremely susceptible to disease because of its impaired immune system, reached a production level of more than a thousand per week.

The farm operations, which provides care for the larger domesticated animals required by several FCRC studies, were evaluated and found to be justified and cost-effective.

Significance to Biomedical Research and the Program of the Institute: The Biological and Chemical Carcinogenesis Programs, together with the Carcinogenesis Intramural Program, have become firmly entrenched scientifically as highly important efforts within DCCP and for the NCI at-large. This has been recognized by NCI authorities and outside expert reviewers alike; there appears to be little or no residual doubt that facilities, resources, and the scientific staff that has been recruited represent an establishment that has and increasingly will continue to make genuine research contributions toward solving the cancer problem.

Increasingly significant experimental results from a variety of investigations on host-virus interactions, on potential interrelationships among viruses and cell transformational events, on biological, biochemical, and serological characteristics of oncogenic viruses and their components, and on vaccine control have been obtained. This has resulted in new pertinent basic and applied information, providing new insights into the elucidation of the relationships of viruses to neoplastic diseases, to their cause, detection, prevention and control. In addition, increased quantities of high quality viruses and viral reagents, much needed both within FCRC and for outside investigators in support of work in virology, immunology, molecular biology and genetics were made available. Along with this, additional program effort was aimed toward the integration of a number of research and testing efforts to assess the risk of cancer in humans from a variety of potential chemical carcinogens. Although it is likely that most chemicals are not carcinogenic, including many which are closely related in structure to compounds known to be carcinogenic, valuable results were obtained in identifying those that are, and in determining carcinogenic potency. Thus, of considerable significance were studies on the bioassay of environmentally important chemicals, development and validation of short term assays, and basic research directed towards understanding mechanisms of carcinogenesis. The latter will point to improved methods of detecting and identifying carcinogens and, perhaps, to

early diagnosis and interruption or reversal of the carcinogenic process, together with steps towards the identification of susceptible individuals.

Proposed Course: As regards future DCCP/FCRC program development, DCCP/NCI may determine that particular research or resource goals require changes in emphasis; objectives may be altered and/or previously established ones postponed or eliminated. Conceptual modifications may be introduced, as for example the recent increased program interest in viruses and chemicals as cocarcinogens. Programs will be reviewed periodically by NCI and expert outside peer review groups and determinations made as to their need or desirability for continuation while new projects may be evaluated for potential program merit and exploratory effort. The continued need for alterations in the conduct of Contractor research will not connote expansion of the current budget. Quite the contrary, advantage will be taken of the fact that obsolete, undesirable, or unproductive program effort(s) are to be phased out. These would be redirected into more fruitful research areas, for example, as described above, with the possible recruitment of outside scientific expertise, but without necessarily elevating personnel levels.

For the immediate future, the DCCP/FCRC Program, while directed toward attainment of essentially the same overall goals and objectives, will continue with important modifications of several of its component parts. In the Biological Carcinogenesis Program, the phase out of the Biology of Type C Viruses Section in the latter part of the current contract year was the result of its being judged as a relatively low priority effort. The same holds for the Endogenous Formation of Carcinogenesis Section in the Chemical Carcinogenesis Program. Moreover, the Endocrine Carcinogenesis Section was eliminated at the end of last year but may undergo some type of revival depending upon availability of suitable professional expertise. In the area of In Vivo Carcinogenesis, efforts with current chemical carcinogens will continue until final commitments have been achieved with respect to standardized testing of the compounds on behalf of the NTP. Facilities and resources will continue to be used, to a greater extent in support of mechanisms of nitrosamine carcinogenesis and, perhaps, other projects with critical animal holding requirements. A similar situation exists within the In Vitro Carcinogenesis Section. This effort will veer from work previously confined to development of assay procedures for chemical carcinogens and begin to probe into mechanisms of action related to chemical transformation of cells.

Both the Biological and Chemical Carcinogenesis Programs were designed to inherently provide strong but flexible scientific mechanisms that allow continued opportunity to enhance program productivity and effectiveness. Maximal use is made of results of previous efforts as a basis for more advanced work, for rapidly capitalizing on new scientific and technological advances, and for seizing new initiatives in previously untried areas of investigation as related to human cancer. As an illustration of this are DCCP's continuing plans to explore and foster where feasible genuine cross-collaboration of

efforts between the Biological and Chemical Carcinogenesis Programs. Scientific cooperation among at least portions of these respective program endeavors should produce a unique blending of program interests and objectives, and provide significant infusions of scientific expertise. This will, in turn, undoubtedly open new research vistas for exploration, portending the attainment of program accomplishment of great importance in the elucidation of problems concerned with cause and prevention of human cancer.

Date Contract Initiated: September 26, 1977

Current Annual Level:

DCCP/FCRC (Full)*	$11,159.7
Other (Partial)**	1,071.6
ISA***	1,913.6
Total	$14,144.9

* Includes complete DCCP/FCRC funding for Biological and Chemical Carcinogenesis Programs, plus Contractor support for on-site NCI Carcinogenesis Intramural Programs. Also includes fixed and available award fees.

** Includes partial DCCP/FCRC budget support for Environmental Control and Animal Production Area

*** Represents DCCP portion of Interdepartmental Support Agreement for services furnished by U.S. Army.

REPORTS ON INTERNATIONAL AGREEMENTS AND INFORMATION EXCHANGE ACTIVITIES

(Fiscal Year 1980)

The Division of Cancer Cause and Prevention participates actively in a number of international agreements and information exchange activities.

International Agreements

US-USSR Agreement on Health Cooperation - Joint Working Group on Cancer Virology

US-France Cooperation in Cancer Research - Basic Cancer Research Group

US-Germany Cancer Program - Environmental Carcinogenesis

US-Italy Bilateral Agreement - Committee on Cancer Prevention

US-Japan Cooperative Cancer Program (see OIA, NCI report)

US-Peoples Republic of China Health Agreement - Cancer Epidemiology (see OIA, NCI report)

Information Exchange Activity

International Cancer Research Data Bank (see separate section, this report)

US-USSR Agreement. A Memorandum of Understanding for cooperation in the study of the microbiology, immunology, and molecular biology of cancer viruses was first signed on November 18, 1973. The Memorandum established procedures for joint studies through the exchange of information, materials and scientists between the two countries. (Previous delegation meetings: November 1972 Moscow, USSR; November 1973 Bethesda, Maryland USA [Subcommittee]; May 1974 Moscow, USSR; May 1975 Bethesda, Maryland USA; June 1976 Sukhumi, USSR; October 1977 Bethesda, Maryland USA; September 1978 Riga, Latvian SSR; May 1980 Warrenton, Virginia USA)

The seventh meeting of the US-USSR Working Group on Cancer Virology, Co-Chairmen Dr. Louis R. Sibal and Prof. Boris A Lapin, took place at Airlie House, Warrenton, Virginia USA on May 21-23, 1980. Following introduction of the members and an overview of the Cooperation, a scientific symposium was held on the afternoon of the first day and the morning of the second day. Members of both delegations and invited speakers presented reports on recent studies on the role of viruses in primate neoplastic disease and the search for human tumor viruses or viral information. Later, the members reviewed progress of the current cooperation, communicated experiments of mutual interest and began writing the Memorandum of Understanding. The third day was devoted to completing and signing the Memorandum of Understanding.

Both Sides agreed to continue collaborative studies in the following specific areas: (1) studies of viruses isolated from human tissues, in cell culture or

in animals and their possible role in the pathogenesis of human neoplasia; (2) continuation of studies on primate viruses as they relate to human cancer; (3) studies on the role of viruses in the induction of human breast tumors, including continuation of studies on MP-MV and related viruses; (4) studies on co-carcinogenesis, including viruses and other biological agents, chemical and physical factors; (5) characterization of nucleic acids and their role in the induction of animal and human cancers: detection of transforming sequences, their integration and mechanisms leading to transformation; (6) studies on oncogenic viruses important to human ecology: for example, viruses isolated from bovine, avian, feline species; (7) studies using techniques of recombinant DNA research for investigation of the malignant process.

Recognizing that great strides have been made in the past few years on the study of oncogenic viruses, both Sides recommended that these studies be continued using existing and new techniques such as recombinant DNA and hybridoma formation. Both Sides recognized the importance of continuing the exchange of scientists to conduct joint research and participating in conferences, seminars and other scientific sessions on problems of cancer virology.

The composition of the working groups is as follows:

US Delegates	USSR Delegates
Dr. Louis R. Sibal, Chairman National Cancer Institute Bethesda, Maryland	Prof. Boris A. Lapin, Chairman Inst. Exptl. Pathology & Therapy Sukhumi
Dr. Stuart A. Aaronson National Cancer Institute Bethesda, Maryland	Prof. Tomas I. Tikhonenko D.I. Ivanovsky Inst. of Virology Moscow
Dr. J. Thomas August Johns Hopkins University Baltimore, Maryland	Dr. Vladimir I. Strouk Lab. of the Institute of Problems of Oncology Ukraine, Kiev
Dr. Robert M. McAllister Children's Hospital of Los Angeles Los Angeles, California	Dr. Kira B. Fomina D.I. Ivanovsky Inst. of Virology Moscow
Dr. Harvey Rabin Frederick Cancer Research Center Frederick, Maryland	
Dr. Fred Rapp Pennsylvania State University M.S. Hershey Medical Center Hershey, Pennsylvania	
Dr. David Yohn Ohio State University Columbus, Ohio	

Future Meetings: It was proposed that the eighth meeting be held in the USSR in the Autumn 1981. In the interim, small groups of scientists could convene, as needed, to discuss important problems.

The following scientists participated in the exchange program during fiscal year 1980:

USA Scientists to USSR

Scientist/Affiliation	Time / Man Months	Sponsoring Institution(s)
Dr. Seymour Kalter Southwest Fdn. for Research & Education San Antonio, Texas	May 1980 (1/2 month)	D.I. Ivanovsky Inst. (Moscow); Inst. of Expt'l Pathology and Therapy (Sukhumi)
Dr. Ursula Heine National Cancer Inst. Bethesda, Maryland	September 1980 (1/2 month)	Gamaleya Institute (Moscow); Oncologic Scientific Center (Leningrad); Inst. of Cytology (Leningrad); Inst. of Experimental Pathology and Therapy (Sukhumi)

USSR Scientists to USA

Prof. Boris A. Lapin Inst. of Experimental Pathology and Therapy Sukhumi, USSR	May 1980 (1/2 month)	Seventh Meeting of US-USSR Joint Working Group on Cancer Virology
Prof. T. I. Tikhonenko D.I. Ivanovsky Inst. of Virology	May 1980 (1/2 month)	Seventh Meeting of US-USSR Joint Working Group on Cancer Virology
Dr. Vladimir I. Strouk Lab. of the Institute of Problems of Oncology	May 1980 (1/2 month)	Seventh Meeting of US-USSR Joint Working Group on Cancer Virology
Dr. Kira B. Fomina D.I. Ivanovsky Inst. of Virology	May 1980 (1/2 month)	Seventh Meeting of US-USSR Joint Working Group on Cancer Virology

US-France Cooperation. During fiscal year 1980, there were no joint meetings of the Viral Oncology Delegation of the US-France Cancer Program. However, in December 1979, a committee composed of NIH (NCI) and INSERM officials met to review the progress of the Cooperation. Both Sides were satisfied by the scientist exchanges; but the French indicated that they were considering reorganizing the Working Groups, reducing the number from three to two. Several months later, under the new INSERM Directeur Generale, Professor Philippe Laudat, the French formed a clinical research and a basic research

working group. At a meeting in Paris in December 1979, Dr. Vincent DeVita, Acting Director, NCI, agreed in principle to this reorganization and combined the U.S. Viral Oncology and Hormone/Cancer Working Groups to form one group which would be responsible for basic research related to cancer. Dr. Louis Sibal, DCCP, NCI, then head of the viral oncology group was appointed Chairman of the U.S. working group and, similarly, Dr. Jean-Paul Levy, Hopital Cochin, Paris, was appointed Chairman of the French group.

During this period of reorganization, both working groups adopted a "business as usual" policy but the changes, particularly in the categories of research activities undertaken and the conditions of the exchange, have not been redefined. In April 1980, the two chairmen met to discuss (1) the scope or range of projects to be supported under this Cooperative Agreement and (2) the conditions for the selection of projects, investigators and length of support.

There was general agreement to call the group "Recherches Fundamentaux sur la Cancerogenese" ("Basic Research in Carcinogenesis") and complete accord that any nonclinical study of scientific merit relating to the process of carcinogenesis could be considered for support. Some examples of the research areas which could be supported are: cell proliferation, cell growth factors, cancer cell and normal cell differentiation, molecular genetics, tumor promotors, DNA repair, induction of transformation by DNA viruses, RNA viruses, chemical agents, and physical agents, etc.

Tentatively, the Chairmen agreed to accept scientist exchanges for varying periods of time, thus permitting the achievement of a variety of specific goals. However, unusually short (1-2 weeks) or unusually long (1 year) periods, extensions, and return visits would require strong justification. Periods of support of 3-6 months would be considered optimal.

The composition of the working groups is as follows:

US Delegates	French Delegates
Dr. Louis R. Sibal, Chairman National Cancer Institute Bethesda, Maryland	Prof. J. P. Levy, Chairman Hopital Cochin Paris
Dr. Howard Bern Univ. of California, Berkeley Berkeley, California	Prof. Francois Cuzin Centre de Biochimie Nice
Dr. Eugene DeSombre University of Chicago Chicago, Illinois	Prof. Michel Daune Inst. de Biologie Moleculaire Strasbourg
Dr. Raymond V. Gilden Frederick Cancer Research Center Frederick, Maryland	Prof. Claude Helene Museum-National d'Histoire Naturelle Paris

Dr. Veronica Maher
Michigan State University
East Lansing, Michigan

Prof. Roger Monier
Inst. de Recherches
Scientifiques sur le Cancer
Villejuif

Dr. Fred Rapp
Pennsylvania State University
M.S. Hershey Medical Center
Hershey, Pennsylvania

Prof. H. Rochefort
Centre de Recherches de l'INSERM
Montpellier

Dr. Edward M. Scolnick
National Cancer Institute
Bethesda, Maryland

Dr. D. Stehelin
Unite de Recherches de Virologie
Moleculaire
Lille

Dr. Stuart Yuspa
National Cancer Institute
Bethesda, Maryland

Dr. Andree Tixier-Vidal
College de France
Paris

Prof. Laudat officially invited the U.S. working group to participate in the first joint working meeting on Recherche Fundamentaux sur la Carcinogenesis at INSERM Headquarters, Paris, for 2 days during the week of October 27, 1980.

Because of the reorganization, exchanges during fiscal year 1980 were delayed to the latter part of the year. Several actions on both Sides are being reviewed for scientific merit at the time of this report. Nevertheless, the exchanges show signs of becoming active and productive. The following table summarizes the exchanges during fiscal year 1980.

USA Scientists to France

Scientist/Affiliation	Time / Man Months	Sponsoring Institution(s)
Dr. George Fareed Univ. of California, Los Angeles, California	April 1980 (4 months)	University of Nice
Dr. Carlos Sonnenschein Tufts University Boston, Massachusetts	May 1980 (3/4 month)	Universite de Paris
Dr. Maurice Cohen Frederick Cancer Research Center Frederick, Maryland	August 1980 (1/2 month) (approval pending)	Hopital Cochin, Paris
Dr. Carlo Moscovici VA Hospital Gainesville, Florida	September 1980 (3 months) (approval pending)	Inst. of Experimental Embryology, Paris
Dr. Robert Grafstrom Johns Hopkins University Baltimore, Maryland	September 1980 (2 months) (approval pending)	Inst. Gustave Roussy Villejuif

French Scientists to USA

Dr. Georges Calothy Foundation Curie- Inst. du Radium	November 1979 (6 months)	Rockefeller University New York, New York
Dr. Catherine Guibout INSERM, Paris	December 1979 (2 months)	Duke University Durham, North Carolina
Dr. Gerard Goubin Inst. Curie, Orsay	August 1980 (6 months) (approval pending)	Sidney Farber Cancer Center Boston, Massachusetts
Dr. Marc Fellous Hopital St. Louis Paris	August 1980 (1 month) (approval pending)	Harvard University Cambridge, Massachusetts
Ms. Evelyne Sage Centre de Biophysique Moleculaire, Orleans	October 1980 (3 months) (approval pending)	Albert Einstein College of Medicine New York, New York
Dr. Evelyne Nahon-Merlin Inst. Gustave Roussy Villejuif	6 months (approval pending)	Columbia University New York, New York

US-Italy Cancer Program. At a meeting in April 1979, of scientists from the US and Italy agreed to collaborate in the following areas: (1) asbestos; (2) immunopharmacology; (3) biological studies in breast cancer; (4) occupational cancer studies; (5) mechanisms of transformation; (6) consultation and training in epidemiology. The joint working committee agreed that studies in these areas be implemented through exchange visits, workshops and collaborative projects in a manner similar to the other bilateral agreements.

Recently, Dr. Gregory T. O'Conor was appointed as Chairman of a Committee on Cancer Prevention. The first joint meeting is planned for October 1980 in Milan, Italy at which time the Group will define the purpose of sponsored joint conferences and workshops, the selection of exchange scientists and the exchange of materials and resources. A Memorandum of Understanding for this Agreement will be developed in October 1980; 18-20 man months are anticipated for scientist exchanges.

The Committee is comprised of the following members:

US Delegates	Italian Delegates
Dr. Gregory T. O'Conor, Chairman National Cancer Institute Bethesda, Maryland	Dr. Leonardo Santi, Chairman Inst. Scientifico per le Studio e la cura dei tumori Genova
Dr. Fred Becker M.D. Anderson Hosp. & Tumor Inst. Houston, Texas	Dr. Giuseppe Della Porta Inst. Nazionale per lo Studio e la cura dei tumori Milano

Dr. William J. Blot
National Cancer Institute
Bethesda, Maryland

Dr. W. Thomas London
Fox Chase Cancer Center
Philadelphia, Pennsylvania

Dr. K. Robert McIntire
National Cancer Institute
Bethesda, Maryland

Dr. Robert C. Nowinski
Fred Hutchinson Cancer Research Ctr.
Seattle, Washington

Dr. Benedetto Terracini
Inst. di Anatomia Patologica Univ
Torino

Dr. Cesare Maltoni
Centro Medico Scoiale per la
prevenzione e lo Studio
dei tumori
Bologna

Dr. Giancarlo Maltoni
Centro Medicina Preventiva
Firenze

Dr. Luigi Chieco Bianchi
Inst. di Anatomia Patologica
Padova

Dr. Nicola Loprieno
Inst. di Antropologia
Pisa

The following scientist exchange is proposed during this fiscal year:

Italian Scientist to USA

Scientist/Affiliation	Time / Man Months	Sponsoring Institution
Dr. Fausto Titti Inst. Superiore di Sanita Rome	1 year (approval pending)	Mt. Sinai School of Medicine New York, New York

US-Germany Cancer Program. At a meeting held in June 1979, at Mainz, Germany two groups of scientists headed by Dr. Richard Griesemer (National Toxicology Program - USA) and Professor F. Oesch, (University of Mainz, Federal Republic of Germany) agreed to promote cooperation on the long-term and short-term testing of mutagenic and carcinogenic substances. When the National Toxicology Program was placed under the aegis of the National Institute of Environmental Health Services, NCI decided to develop a bilateral cooperation in carcinogenesis, an area in which NCI had greater interest and expertise.

A working group was established to explore the possibilities of a US-German Cooperation in the areas of mechanism of carcinogenesis, in vitro test development, occupational cancer and epidemiology. On April 17, 1980 both Sides met in Heidelberg, Germany and prepared a detailed Memorandum of Understanding for consideration by the Acting Director, NCI, and the Minister of Research and Technology, Federal Republic of Germany, outlining two broad areas for cooperation in the field of carcinogenesis: (1) causation and mechanisms in carcinogenesis, including evaluation and validation of methods in carcinogenesis research; and (2) prevention and modulation of the

carcinogenic process. The joint expertise for these projects is available in both countries. Both Sides agreed to exchange of scientists, limited to 24 man months; exchange of materials; and exchange of scientific information, including unpublished data.

In addition, both Sides agreed on the the need for consultative committees to evaluate applications of scientists who wish to travel to foreign laboratories.

The composition of these working groups is as follows:

US Delegates	German Delegates
Dr. Joseph A. DiPaolo, Chairman National Cancer Institute Bethesda, Maryland	Prof. D. Schmahl German Cancer Research Center Heidelberg
Dr. Elizabeth Miller McArdle Laboratories Madison, Wisconsin	Prof. F. Oesch University of Mainz
Dr. Philippe Shubik Eppley Institute Omaha, Nebraska	

US-People's Republic of China Health Protocol. Although this Agreement has not been fully developed, it is proposed that during November 1980 a delegation on epidemiology consisting of Dr. Joseph F. Fraumeni, Jr., NCI, Leader; Dr. William J. Blot, NCI; Dr. Frederick P. Li, NCI; Dr. Robert W. Miller, NCI; and Dr. Earl S. Pollack, NCI, travel to the People's Republic of China. (See OIA, NCI report for recent progress on this Agreement.)

SUMMARY REPORT

INTERNATIONAL CANCER RESEARCH DATA (ICRDB) PROGRAM

October 1, 1979 through September 30, 1980

Mission

The ICRDB Program was established by the National Cancer Act of 1971, in recognition of the urgent need for a comprehensive, large-scale effort to collect and disseminate the massive amount of information resulting from increased support for cancer research. The ICRDB Program represents a relatively small investment of NCI resources intended to help maximize the return to society on the large investment in research.

Because the volume of cancer literature far surpasses the reach of individual scientists, there is danger that much of the potential benefit from research might be lost where investigators remain unaware of new findings relevant to their own experiments. The ICRDB Program has developed an effective multi-faceted system for promoting the rapid exchange of cancer research findings among scientists so that this information can be most productively utilized.

Organizational Changes

In accordance with recommendations of the Reorganization Task Force chaired by Dr. William Terry, the Director of the National Cancer Institute in 1979 approved the transfer of the ICRDB Program from the Office of International Affairs, OD, to the Office of the Director, Division of Cancer Cause and Prevention. This decision reflected the majority opinion of the Task Force that the ICRDB Program was working well, with the support and participation of Dr. O'Conor an important element, and that it would be desirable to continue this working relationship by relocating the ICRDB Program within the Division of which he was Director.

Accomplishments

To facilitate the transfer of cancer research information among scientists, the ICRDB Program has: 1) developed three on-line computer data bases which enable scientists to easily retrieve cancer information at more than 1,100 locations within the United States and in 13 other countries; 2) developed several series of publications providing complete coverage of cancer research information, in special formats designed for easy use and quick reference; and 3) supported a variety of specialized information collection, analysis and dissemination activities. These services and activities are described briefly below.

Computer Data Bases - The CANCERLINE System:

CANCERLIT: Contains more than 200,000 abstracts of published cancer literature, papers presented at meetings, books, technical reports and theses;

CANCERPROJ: Contains descriptions of 17,500 current cancer research projects from 75 countries; and

CLINPROT: Contains summaries of some 2,000 experimental cancer treatment protocols.

CANCERLIT is growing at a rate of nearly 40,000 abstracts per year, screened from over 3,000 biomedical journals. Because of stringent input processing requirements and monthly updating of CANCERLIT, the most recently published research results are rapidly made available to cancer researchers worldwide. As of early 1980, all new entries to CANCERLIT are indexed with the MeSH vocabulary developed by the National Library of Medicine, making them easier to retrieve via on-line searching. ICRDB screening, indexing and abstracting activities are performed under contract by the Franklin Research Center.

CANCERPROJ offers the most comprehensive available source for ongoing cancer research project information, including nearly 3,200 project descriptions collected by an international network of data input coordinators. CANCERPROJ is updated quarterly. Collection, input and updating of project descriptions are performed by the Current Cancer Research Project Analysis Center (CCRESPAC) under an inter-agency agreement with the Smithsonian Science Information Exchange.

CLINPROT provides worldwide access to summaries of new clinical cancer treatment protocols currently being evaluated at major American and foreign cancer centers. CLINPROT is updated quarterly. Collection, input and updating of protocols are performed under contract by Informatics, Inc.

The CANCERLINE data bases are made available worldwide through the National Library of Medicine (NLM) computer system, supported by an inter-agency agreement. Foreign access CANCERLINE has been enhanced by 1) the recent addition of CANCERLINE to the EURONET system in Europe, and 2) a collaborative project with the British Library which provides free CANCERLINE services to nearly 100 developing countries (see Special Information Activities, below).

ICRDB Publications:

CANCERGRAMS: Monthly bulletins containing abstracts of recently published literature in 65 major cancer research areas;

Special Listings of Current Cancer Research: Annual compilations of ongoing research projects in 50 different cancer research areas;

ONCOLOGY OVERVIEWS: Retrospective bibliographies with abstracts on 30 topics per year selected for high current interest to cancer researchers;

Compilation of Cancer Therapy Protocol Summaries;

Directory of Cancer Research Information Resources; and

Special collaborative publications (See Special Information Activities, below).

CANCERGRAMS are prepared monthly from the newest input to the CANCERLIT data base by scientists at three Cancer Information Dissemination and Analysis Centers (CIDACS; see Special Information Activities, below) and a network of nearly 100 researcher-consultants, and rapidly disseminated to nearly 10,000 scientists worldwide. Containing abstracts screened from over 3,000 biomedical journals, CANCERGRAMS constitute an effective "handle" on the vast cancer literature.

Special Listing of Current Cancer Research are extracts from the CANCERPROJ data base, prepared by scientists at the CCRESPAC (see Special Information Activities, below). They are intended to stimulate interaction between investigators pursuing related avenues of research.

ONCOLOGY OVERVIEWS are extracts from the CANCERLIT data base covering a period of several years, providing in-depth coverage of emerging foci of cancer research. OVERVIEWS are prepared by scientists at the CIDACs, with review and editorial commentary by well-known researchers in each topic area. They provide an excellent means of rapidly updating knowledge in burgeoning areas of research.

The Compilation of Cancer Therapy Protocol Summaries (4th Edition, April 1980) is derived from the CLINPROT data base and contains over 1,900 protocol entries. New features of this edition include 1) the incorporation of all immunotherapy protocols previously published separately as the Compendium of Tumor Immunotherapy Protocols, and 2) a special section separately listing closed protocols. The Compilation is indexed by tumor, agent, and protocol identification number, and is a useful reference for the practicing oncologist. It is prepared under contract by Informatics, Inc.

The Directory of Cancer Research Information Resources (2nd Edition, May 1979) contains over 800 entries covering a wide spectrum of resources available to health professionals. It is prepared under contract by JRB Associates. The 1981 edition is now in preparation.

The ICRDB publications described above are printed and distributed by the National Technical Information Service (NTIS), under an inter-agency agreement.

Special Information Activities:

1. Cancer Information Dissemination and Analysis Centers (CIDACs)

Three contract-supported centers have been established as information resources in the following broad areas of cancer research:

1. Diagnosis and Therapy -- University of Texas, M.D. Anderson Hospital and Tumor Institute
2. Carcinogenesis -- Franklin Research Center; and
3. Cancer Virology, Immunology and Biology -- Franklin Research Center.

Each CIDAC is staffed by scientists and served by a consultant network with special expertise in the appropriate fields. Within their own subject area, each CIDAC prepares CANCERGRAMS and ONCOLOGY OVERVIEWS, performs custom CANCERLINE searches, and provides scientific guidance to the ICRDB Program.

2. Current Cancer Research Project Analysis Center (CCRESPAC)

Operated by the Smithsonian Science Information Exchange, the CCRESPAC collects and processes ongoing research project information, generates the CANCERPROJ data base, prepares the Special Listings of Current Cancer Research, and performs custom data base searches on request.

3. Clearinghouse for On-going Research in Cancer Epidemiology

This cooperative project is supported jointly by the ICRDB Program, the International Agency for Research on Cancer (IARC) in Lyon, France, and the German Cancer Research Center in Heidelberg, Germany. The Clearinghouse, located in Lyon, collects, processes, and disseminates detailed data on research related to cancer epidemiology and studies of human cancer causation in countries around the world. The Clearinghouse also prepares lists of epidemiology researchers and resources, responds to technical questions and produces annually a Directory of On-Going Research in Cancer Epidemiology.

4. Latin American Cancer Research Information Project (LACRIP)

The ICRDB Program, in collaboration with the Pan American Health Organization (PAHO) and its Regional Library of Medicine (BIREME) in Sao Paulo, has developed and implemented mechanisms for identifying, collecting, and supplying Latin American biomedical literature and data about ongoing cancer-related research projects in Latin America for input to the CANCERLINE system.

PAHO also serves as the center for searching ICRDB data bases and providing documents and data in response to requests for information from cancer researchers in Latin America.

Through LACRIP, a series of collaborative clinical studies have been developed between nine cancer centers in the United States and six centers in Latin America. LACRIP maintains clinical data gathered at the Latin American centers and also arranges for the exchange of professional staff between centers in order to promote a better understanding of the current cancer treatments available in the United States.

5. Scientist-to-Scientist Communication

The ICRDB Program, through the International Union Against Cancer (UICC) in Geneva, Switzerland, encourages international scientist-to-scientist communication through two different mechanisms:

International Cancer Research Technology Transfer Program (ICRETT)--This program promotes direct and rapid transfer of information about new or improved technology or methodology between two or more investigators located in different countries. This is accomplished by supporting short-term visits for the purpose of conducting brief collaborative research projects by investigators working in different countries. Since the inception of the program in 1975, 397 ICRETT awards have been granted (through March 1980).

International Cancer Research Workshop Program (ICREW)--This program provides partial support for workshops in cancer research where scientists meet on an international basis. The aim is to increase the frequency, speed, and efficiency of direct information exchange among small groups of researchers active in the same field of basic, clinical, or behavioral research related to cancer. Since the inception of the program in 1975, 59 ICREW awards have been granted (through March 1980).

6. A Project to Promote International Collaboration Between Cancer Research Organizations

In cooperation with the International Union Against Cancer (UICC) in Geneva, support is provided for a special Committee for International Collaborative Activities (CICA), within the framework of the UICC. The Executive Secretary and members of CICA are providing advisory, consultative and liaison services to support the international activities of the United States National Cancer Program, in general, and the ICRDB Program in particular.

CICA aids in the collection of data about ongoing cancer research projects (including clinical protocols) from countries around the world. CICA personnel also identify and promote collaborative projects among cancer centers and cancer scientists in different countries. A CICA project is the publication of a periodically updated International Directory of Specialized Cancer Research and Treatment Establishments, containing descriptions of nearly 700 cancer centers around the world.

An International Cancer Patient Data Exchange System (ICPDES) has been established under CICA sponsorship, and present participants include nine European and five United States cancer centers. The ICPDES could result in the first internationally recognized and standardized tumor registry, providing comparative data of value in cancer prevention and treatment.

7. International Medical Information Center (IMIC)

Under contract to the ICRDB Program, the IMIC in Tokyo coordinates the screening and collection of cancer-related information from Japan and other Asian countries for entry into the CANCERLINE data bases.

8. Cancer Information Service to Developing Countries (CISDC)

The British Library, supported by a contract with the ICRDB Program, provides searches of ICRDB data bases to scientists in developing countries. A limited number of copies of useful articles identified as a result of the CANCERLINE searches is also provided to requestors under this contract.

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BRITISH LIBRARY (NO1-CO-85410)

Title: Cancer Information Services for Developing Countries

Contractor's Project Director: Dr. Philip Holmes

Project Officer (NCI): Donna J. Wicker

Objective: The CISDC project provides free access to the ICRDB collection of cancer information for cancer scientists in developing countries.

Major Accomplishments: In 1979, a major effort was undertaken to identify medical institutions located in 100 Asian and African countries with per capita GNP under $2,500 per year. Following preparation of the list each of the institutions received brochures and literature describing the CISDC Program and explaining procedures for using the service. By the end of the year, after 9 months of operation, approximately 500 search requests had been filled.

Significance to Biomedical Research and Program of the Institute: This project provides access to the comprehensive ICRDB collection for scientists in countries with poor informational facilities. An important feature of the CISDC Program is the provision of 5 photocopies of full articles selected from the bibliographic listing for each search.

Proposed Course: The contract will continue until current funds are expended at which time it will be phased out.

Date Contract Initiated: September 30, 1978

Current Annual Level: $215,602

DEPARTMENT OF COMMERCE (National Technical Information Service) (YO1-CO-60702)

Title: ICRDB Document Announcement and Dissemination Services

Contractor's Project Director: James Jennings

Project Officer (NCI): Dr. Hortencia Hornbeak

Objective: This agreement supports the printing and dissemination of ICRDB publications, the announcement of these documents to potential users, and maintenance of all ICRDB documents in archival storage for supplying copies on request.

Major Accomplishments: Currently, NTIS disseminates more than 23,000 copies of CANCERGRAMs per month to more than 10,000 investigators. In addition 50 Special Listings of Current Cancer Research and 30 ONCOLOGY OVERVIEWs are published yearly. An average of 800 copies of each Special Listing and 500 copies of each ONCOLOGY OVERVIEW are distributed to

investigators. The annual Compilation of Cancer Therapy Protocol Summaries is disseminated to over 6,000 investigators. NTIS also distributes 5,000 copies of other ad hoc ICRDB publications yearly. NTIS announcements have resulted in over 2,000 additional subscriptions to CANCERGRAMs.

Significance to Biomedical Research and Program of the Institute: This interagency agreement has allowed the ICRDB Program to rapidly fulfill one of its mandated activities, namely, the broad dissemination of biomedical research information on cancer. Through its various worldwide outlets, NTIS performs a valuable service in disseminating ICRDB products to the global scientific community.

Proposed Course: Plans call for the continuation of the agreement through September 29, 1980.

Date Agreement Initiated: September 30, 1976

Current Annual Level: $418,400

DEPARTMENT OF COMMERCE (National Technical Information Service, Smithsonian Science Information Exchange, SSIE) (YO1-CO-00708)

Title: Current Cancer Research Project Analysis Center

Contractor's Project Director: Dr. Donald Elliott

Project Officer (NCI): Dr. Hortencia Hornbeak

Objective: The overall objective of this agreement is the operation of a Current Cancer Research Project Analysis Center (CCRESPAC) to support the International Cancer Research Data Bank (ICRDB) Program by coordinating the collection and processing of research project descriptions.

Major Accomplishments: The contractor prepares for publication fifty annually updated Special Listings of Current Cancer Research, which consist of edited summaries of on-going research in specific cancer subject areas. Computer tapes containing over 17,500 cancer projects are prepared on a quarterly basis for the regeneration of the CANCERPROJ data base, which is available for on-line searching and retrieval via the NLM MEDLARS computer system. Continued correspondence and other communications with scientists and clinicians in 75 countries has resulted in the addition of over 3,200 non-U.S. research projects to the CCRESPAC information system. The tasks of preparing and updating clinical protocol summaries have been transferred from this interagency agreement to a separate contract awarded to Informatics, Inc. on December 15, 1979.

Significance to Biomedical Research and Program of the Institute: This interagency agreement promotes the exchange of cancer research project information among researchers around the world by providing a variety of information products and services tailored to their individual needs. Current awareness of research in progress enables investigators to effectively utilize new concepts and techniques in the design of their own research projects.

Proposed Course: SSIE is presently being incorporated into NTIS, Department of Commerce. This transition will be officially completed as of October 1, 1980.

Date Agreement Initiated: December 30, 1974

Current Annual Level: $419,399

FRANKLIN RESEARCH CENTER (NO1-CO-05463)

Title: Screening, Indexing and Abstracting of Published Cancer-Related Literature

Contractor's Project Director: Dr. Bruce H. Kleinstein

Project Officer (NCI): Donna J. Wicker

Objectives: The SIA project collects, abstracts and indexes cancer literature published in professional journals, monographs and reports. This literature collection is the source material for the three primary services of the ICRDB Program: CANCERLIT, CANCERGRAMs, and ONCOLOGY OVERVIEWs.

Major Accomplishments: Approximately 4,000 items are photocopied, abstracted, indexed and keyed onto magnetic tape each month and sent to the ICRDB computer contractor for the final reformatting required to update the computer data base. The items are collected from over 3,000 journals, and are abstracted from articles written in over 20 languages within 30 days of the receipt of each journal issue.

Significance to Biomedical Research and Program of the Institute: The SIA project is a multifaceted activity that provides rapid scanning of the world's cancer literature followed by processing that permits rapid, easy access to narrow cancer topics within a fraction of the time a scientist would normally have to spend retrieving the same material in a library. Access to the complete collection is possible via the CANCERLIT data base or, following further processing, via the secondary publications ICRDB produces in narrow cancer subject areas.

Proposed Course: The contractor will continue the activity as described. Plans for 1980 include improvement of the collection through stricter quality controls.

Date Contract Initiated: June 30, 1977

Current Annual Level: $1,537,340

FRANKLIN RESEARCH CENTER (NO1-CO-85403)

Title: Cancer Information Dissemination and Analysis Center (CIDAC) for Chemical Environmental and Radiation Carcinogenesis

Contractor's Project Director: Dr. L. Gerald Parchman

Project Officer (NCI): Dr. Dianne E. Tingley

Objectives: The CIDAC provides scientific input necessary to produce information products and services for cancer researchers, and provides guidance to the ICRDB Program, in the area of carcinogenesis.

Major Accomplishments: The CIDAC regularly produces 20 monthly CANCERGRAMs, current awareness bulletins containing abstracts of recently published literature. A new CANCERGRAM series on "Short-term Test Systems for Carcinogenicity and Mutagenicity" was initiated in 1979. Ten ONCOLOGY OVERVIEWs, retrospective bibliographies with abstracts concerning high interest topics in carcinogenesis research, are published annually. Recently covered topics include the roles of artificial sweeteners, alcohol, vinyl chloride, water supply contaminants, and genetic predisposition in carcinogenesis. The CIDAC performs custom searches of the CANCERLINE data bases in response to requests for information; submits monthly Highlight Reports, pinpointing significant new developments in carcinogenesis research; and assists in data base quality control.

Significance to Biomedical Research and Program of the Institute: The CIDAC serves as a valuable resource for the NCI and the worldwide cancer research community in the area of carcinogenesis. The CANCERGRAMS collectively provide comprehensive coverage of this entire field, quickly alerting researchers to new findings with minimal expenditure of effort, and thereby allowing them more time for productive research. ONCOLOGY OVERVIEWs enable researchers to rapidly update their knowledge in emerging areas of research concentration.

Proposed Course: The contractor will continue production of CANCERGRAMs and ONCOLOGY OVERVIEWs and provision of information services. Plans are in preparation for a symposium on topics in chemical carcinogenesis, including possibilities for videotaping and subsequent distribution.

Date Contract Initiated: May 4, 1978

Current Annual Level: $230,341

FRANKLIN RESEARCH CENTER (NO1-CO-85404)

Title: Cancer Information Dissemination and Analysis Center (CIDAC) for Cancer Virology, Immunology and Basic Cancer Biology (VIB)

Contractor's Project Director: Dr. Jack Harris

Project Officer (NCI): Dr. Dianne E. Tingley

Objectives: The CIDAC provides scientific input necessary to produce information products and services for cancer researchers, and provides guidance to the ICRDB Program, in the area of cancer virology, immunology, and basic cancer biology.

Major Accomplishments: The CIDAC regularly produces 24 monthly CANCERGRAMs, current awareness bulletins containing abstracts of recently published literature. Ten ONCOLOGY OVERVIEWs, retrospective bibliographies with abstracts concerning high interest topics in basic cancer research, are published annually. Recently covered topics include cell hybridization, fibronectin, retinoids, transfer factor and immune RNA. The CIDAC performs custom searches of the CANCERLINE data bases in response to requests for information; submits monthly Highlight Reports, pinpointing significant new developments in basic cancer research; and assists in data base quality control.

Significance to Biomedical Research and Program of the Institute: The CIDAC serves as a valuable resource for the NCI and the worldwide cancer research community in the area of cancer virology, immunology, and biology. The CANCERGRAMS collectively provide comprehensive coverage of this entire field, quickly alerting researchers to new findings with minimal expenditure of effort, and thereby allowing them more time for productive research. ONCOLOGY OVERVIEWs enable researchers to rapidly update their knowledge in emerging areas of research concentration.

Proposed Course: The contractor will continue production of CANCERGRAMs and ONCOLOGY OVERVIEWs and provision of information services. Plans are in preparation for a workshop on mechanisms of metastasis, including possibilities for videotaping and subsequent distribution.

Date Contract Initiated: June 22, 1976

Current Annual Level: $299,514

IIT RESEARCH INSTITUTE (NO1-CO-55221)

Title: Computer Support for Cancer Information Dissemination

Contractor's Project Director: Peter B. Schipma

Project Officer (NCI): Dr. John H. Schneider

Objective: The purpose of the contract is to establish and operate a Computer Support Center (CSC) for the ICRDB Program.

Major Accomplishments: The contractor performs a wide variety of computer operations necessary for the creation and maintenance of ICRDB data bases, preparation of ICRDB publications, maintenance of special mailing lists, statistical reporting and special tasks identified by the Program.

Significance to Biomedical Research and Program of the Institute: The excellent computer support provided by the contractor is of central importance to the entire spectrum of ICRDB products and services, whereby the Program is able to fulfill its mandate to actively promote the dissemination of cancer research information on a worldwide basis.

Proposed Course: This contract expires June 26, 1980. As a result of a recompetition, the IIT Research Institute has been identified as the successful bidder for the next four-year period.

Date Contract Initiated: June 27, 1975

Current Annual Level: $227,547 (six-month funding)

INFORMATICS, INC. (NO1-CO-05509)

Title: Preparation and Updating of Clinical Protocol Summaries

Contractor's Project Director: Marilynn Meinke

Project Officer (NCI): Dr. Hortencia Hornbeak

Objectives: This contract provides the capabilities for collecting, processing and disseminating on-going cancer therapy protocol summaries to clinicians and investigators throughout the world.

Major Accomplishments: Prior to award of this contract, these activities were performed by the CCRESPAC contractor, the Smithsonian Science Information Exchange. Informatics prepared the 1980 edition of the Compilation of Cancer Therapy Protocol Summaries which contained 1,300 active protocols and 620 closed protocols, and included protocol summaries which were previously published as the Compendium of Tumor Immunotherapy Protocols. Magnetic tapes containing the summaries of all protocols were provided for the quarterly regenerations of the CLINPROT on-line data base.

Significance to Biomedical Research and Program of the Institute: Both the Compilation and the CLINPROT data base are valuable information resources for apprising practicing oncologists of the latest developments in cancer treatment research. The availability of the principal

investigator's or group chairman's address and telephone number on all protocol summaries facilitates the interaction of clinicians and investigators with common interests.

Proposed Course: Plans call for the continuation of this contract through December, 1982, including annual updating of the Compilation.

Date Agreement Initiated: December 14, 1980

Current Annual Level: $224,872

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (NO1-CO-55195)

Title: Clearinghouse for On-going Work in Cancer Epidemiology

Contractor's Project Director: Dr. Calum S. Muir

Project Officer (NCI): Dr. John H. Schneider

Objective: This contract provides a special mechanism for intensive collection and dissemination of information about current cancer-related epidemiology projects.

Major Accomplishments: The Clearinghouse, located in Lyon, France, continually identifies and contacts new sources of epidemiology research project descriptions. Project descriptions are collected, edited, and published annually as the Directory of On-going Research in Cancer Epidemiology, as well as provided on magnetic tape for entry into the CANCERPROJ data base. The 4th edition of the Directory (1979) contained 1,092 project descriptions collected from 67 countries.

Significance to Biomedical Research and Program of the Institute: By serving as a resource for epidemiological data and establishing communication among epidemiology researchers worldwide, the Clearinghouse promotes international awareness and cooperation which contributes to more productive research in this area.

Proposed Course: Work will continue as described.

Date Contract Initiated: February 25, 1975

Current Annual Level: $127,129

INTERNATIONAL MEDICAL INFORMATION CENTER (NO1-CO-75362)

Title: Development and Implementation of Mechanisms for Interaction Between the ICRDB Program and Asian Countries.

Contractor's Project Director: Masanobu Fujikawa

Project Officer (NCI): Dr. John H. Schneider

Objective: This project was established to enhance the coverage of Asian cancer literature by the ICRDB Program, and to facilitate communication with Asian cancer researchers.

Major Accomplishments: The contractor provides annually 1,500-2,000 cancer-related abstracts or annotations for input to the CANCERLIT data base, screened from approximately 150 Asian-language journals and proceedings of Asian cancer meetings (mostly Japanese).

Significance to Biomedical Research and Program of the Institute: Through CANCERLIT, this activity makes available to researchers worldwide cancer-related findings which might otherwise pass unnoticed because of the Asian language barrier.

Proposed Course: The contract will continue, with increasing emphasis on meeting abstracts and coverage of non-Japanese Asian literature.

Date Contract Initiated: November 30, 1976

Current Annual Level: $161,865

JRB ASSOCIATES, INC. (NO1-CO-85383)

Title: Technical Support Services for the International Cancer Research Data Bank (ICRDB) Program

Contractor's Project Director: Richard Hildreth

Project Officer (NCI): Dr. J. Wesley Simmons

Objectives: This project provides a broad range of technical support activity to all projects within the ICRDB Program and to related information activities within DCCP.

Major Accomplishments: CANCERGRAM flow analysis studies have contributed to decreasing production time required for CANCERGRAMs, the principal ICRDB publication. Support relating to exhibits at major meetings for cancer researchers (e.g., ASCO and AACR) has contributed significantly to the enhancement of user awareness of the ICRDB Program.

Significance to Biomedical Research and Program of the Institute: This project makes available to the ICRDB Program for short- and long-range special projects, as needed, personnel and expertise in the areas of publications preparation, promotion of user awareness, evaluation of user services, and scientific analysis. This support is essential

to fulfillment of the ICRDB Program mandate for active collection and dissemination of cancer research information.

Proposed Course: The present contract will continue until March 21, 1981, with special efforts devoted to formal evaluation of ICRDB products and services, updating of the Directory of Cancer Research Information Resources, and increasing awareness of ICRDB services at major cancer research institutions.

Date Contract Initiated: March 22, 1978

Current Annual Level: $350,000

NATIONAL ACADEMY OF SCIENCES (NAS) (NO1-CO-65280)

Title: Support for U.S. National Committee on the International Council of Societies of Pathology (ICSP)

Contractor's Project Director: June Ewing

Project Officer: Barry M. Goldfarb

Objectives: This project provides support for an on-going program of the NCI to provide a means for effective participation by pathologists of the United States in all matters of concern to the ICSP. The NAS established the U.S. National Committee to coordinate the policies and activities of the societies in this country participating in the ICSP.

Major Accomplishments: The U.S. National Committee participates in the review of tumor classification standards developed through international collaborative efforts, and works to promote the international acceptance of these standards at conferences attended by pathologists.

Significance to Biomedical Research and Program of the Institute: Worldwide agreement on histologic tumor classification findings is highly desirable, in order to clearly interpret clinical research findings on cancer diagnosis and treatment. The professional, technical and promotional guidance provided by the U.S. National Committee contributes to the scientific excellence of the classification standards and insures their widest possible acceptance.

Proposed Course: ICRDB support will not be renewed following completion of work under the present contract.

Date Contract Initiated: October 15, 1975 (precedent contract initiated September 1974)

Current Annual Level: $15,200

NATIONAL ACADEMY OF SCIENCES (NAS) (NO1-CO-75279)

Title: Support of the U.S.A. National Committee on the International Council of Societies of Pathology (ICSP) and WHO International Reference Centers

Contractor's Project Director: June Ewing

Project Officer (NCI): Barry M. Goldfarb

Objective: This project provides support for an on-going program of the World Health Organization (WHO) to promote world-wide standardization of histological tumor classification for the furtherance of medical treatment, research and education. The program is coordinated by the Cancer Unit of WHO and operates through 23 Collaborating Centers, each working on a different tumor type.

Major Accomplishments: This effort has resulted in a series of 21 publications on the International Histologic Classification of Tumors (IHCT), representing an international consensus on tumor identification and nomenclature. The IHCT series is published in 4 languages and distributed worldwide.

Significance to Biomedical Research and Program of the Institute: The IHCT series is designed to facilitate the worldwide adoption of a uniform and standard nomenclature for cancer, without which results of research on cancer diagnosis and treatment cannot be effectively compared.

Proposed Course: Four additional IHCT volumes are now in preparation. ICRDB support of this project will not be renewed following completion of work under the present contract.

Date Contract Initiated: January 16, 1977 (precedent contract initiated September 1974)

Current Annual Level: $6,900

NATIONAL ACADEMY OF SCIENCES (NAS) (NO1-CO-75384)

Title: Support of Activities of the U.S.A. National Committee for the International Union Against Cancer (UICC)

Contractor's Project Director: June Ewing

Project Officer (NCI): Barry M. Goldfarb

Objective: This contract serves the dual purposes of: 1) providing for one representative body (acting on behalf of the various U.S. cancer organizations and foundations) to deal with issues and policies on the

International Union Against Cancer (UICC); and 2) supporting Committee participation in the UICC-sponsored International Cancer Congress held every four years.

Major Accomplishments: Much of the groundwork and planning for the XIII International Cancer Congress to be held in Seattle in 1982 has been completed.

Significance to Biomedical Research and Program of the Institute: This contract supports the representative body which develops and presents the issues and policies of the United States cancer research community to the UICC, and through support of International Cancer Congresses promotes the productive exchange of research information among researchers throughout the world.

Proposed Course: Planning efforts for the International Cancer Congress will continue.

Date Contract Initiated: April 1, 1977

Current Annual Level: $24,990

NATIONAL LIBRARY OF MEDICINE (1-YO1-CO-50003)

Title: Joint NLM/NCI International Cancer Research Data Bank

Contractor's Project Director: Dr. Henry M. Kissman

Project Officer (NCI): Donna J. Wicker

Objective: The agreement with the NLM permits the generation, maintenance and operation of the three ICRDB data bases (published literature, research projects, clinical protocols) on the NLM computer, and for the dissemination of the information in these collections to institutions subscribing to the NLM computer services.

Major Accomplishments: The NLM currently maintains approximately 210,000 abstracts of published literature, 17,500 descriptions of research projects and 2,000 summaries of clinical protocols for the ICRDB Program. Each year a total of 20 updates or regenerations are performed to update or correct the records in these three files. Over 10,000 searches were run against CANCERLINE in 1979.

Significance to Biomedical Research and Program of the Institute: In consonance with the National Cancer Act of 1971, this interagency agreement has given the ICRDB Program the cost savings benefit of using an existing organization with capabilities to reformat, process, and make the results of cancer research available to more than 1,200 locations throughout the world via an existing telecommunications network resident at NLM. These locations include medical schools, medical research

institutions, regional medical libraries and hospitals throughout the United States, and several countries outside the United States. Proposed Course: Plans call for the continuation of this agreement through September 1983.

Date Contract Initiated: July 1, 1974

Current Annual Level: $340,000

PAN AMERICAN HEALTH ORGANIZATION (NO1-CO-65332)

Title: Latin American Cancer Research Information Program

Contractor's Project Director: Dr. Jorge Litvak

Project Officer (NCI): Dr. Hortencia Hornbeak

Objective: This contract established a Latin American Cancer Research Information Program (LACRIP), administered through the Pan American Health Organization (PAHO), which coordinates the collection of journal articles, meeting abstracts, clinical protocols and cancer research projects from Latin America. In addition, LACRIP identifies areas of cancer research that could lead to collaborative efforts with United States investigators; identifies Latin American institutions involved in cancer-related activities and disseminates cancer-related information in Latin America.

Major Accomplishments: The contractor has supplied several hundred cancer-related articles and meeting abstracts from Latin America for inclusion in CANCERLIT. There has been a steady input of cancer research projects and clinical protocols for inclusion into the CANCERPROJ and CLINPROT data bases respectively. At present there are over 160 cancer research summaries from Latin America in CANCERPROJ. The Collaborative Cancer Treatment Research Program (CCTRP) presently sponsors 28 active protocol collaborations involving over 700 patients. The CCTRP has been received with great enthusiasm in Latin America and has stimulated the cooperation of 45 other Latin American cancer institutions and 92 other investigators, at no expense to the ICRDB Program.

Significance to Biomedical Research and Program of the Institute: LACRIP is an important resource for the ICRDB Program in that it supplies project descriptions, clinical protocols and journal articles for the data bases (CANCERPROJ, CLINPROT and CANCERLINE) that would otherwise not be included, and provides a centralized mechanism for dissemination of cancer-related information to a large number of countries.

Proposed Course: The CCTRP will be removed from the work scope at the end of the present contract (July 1981). Since the collaboration has been successful, it is anticipated that DCT will support this program in the future.

Date Contract Initiated: May 24, 1976

Current Annual Level: $541,238

UNION INTERNATIONALE CONTRE LE CANCER (UICC) (NO1-CO-65341)

Title: International Scientist-to-Scientist Information Exchange Program

Contractor's Project Director: Dr. John F. Delafresnaye

Project Officers (NCI): Barry M. Goldfarb, Dr. Robert R. Omata

Objective: The purpose of this program is to promote direct and rapid person-to-person transfer of information about new or improved technology or methodology between investigators from different countries who are working in areas of basic, clinical or behavioral research, in order to further the progress of cancer research.

Major Accomplishments: This contract provides two special mechanisms for promoting international cancer research collaboration: 1) International Cancer Research Technology Transfer (ICRETT) awards enable two cancer researchers from different countries to jointly carry out brief research projects; and 2) International Cancer Research Workshop (ICREW) awards support workshops for direct exchange of research information among scientists. From the inception of these programs through March 1980, 397 ICRETT awards (average period sponsored, 3 weeks) and 59 ICREW awards (average number of participants, 15) have been granted. A majority of ICRETT scientists visited laboratories in the United States and the United Kingdom.

Significance to Biomedical Research and Program of the Institute: Scientists are afforded the valuable opportunity for on-the-spot collaboration necessary for comparing the results of parallel or related research and developing or improving techniques. These interactions frequently lead to continuing exchange of research information, which in turn leads to a more productive collaborative effort.

Proposed Course: Although the ICRETT and ICREW programs are considered worthwhile, this contract activity will not be renewed upon expiration in December 1980, reflecting a re-evalution of ICRDB Program needs and priorities.

Date Contract Initiated: December 4, 1975

Current Annual Level: $602,076

UNION INTERNATIONALE CONTRE LE CANCER (UICC) (NO1-CO-75377)

Title: Liaison and Implementation Projects in Support of the NCI, International Cancer Research Data Bank (ICRDB) Program

Contractor's Project Director: Dr. G. P. Warwick

Project Officer (NCI): Barry M. Goldfarb

Objective: Through its Committee on International Collaborative Activities (CICA), the UICC provides liaison and implementation projects in support of the International Cancer Research Data Bank (ICRDB) Program.

Major Accomplishments: Through 1979, this effort has resulted in collection and processing of information on over 6,000 unpublished current cancer research projects. This information is made available worldwide through the CANCERPROJ data base. The CICA has also established, as a pilot program, an International Cancer Patient Data Exchange System (ICPDES) whereby information covering the entire patient care spectrum is collected in an internationally standardized manner, with participation by 5 major U.S. and 9 foreign cancer centers. As of April 1980, over 11,000 case reports had been entered, covering breast, colorectal and laryngeal cancers, and Hodgkin's and non-Hodgkin's lymphomas. In 1979, the CICA catalyzed the formation of the Organization of European Cancer Institutes (OECI).

Significance to Biomedical Research and Program of the Institute: CICA activities have been of major significance in making the ICRDB Program truly international in scope. Active efforts to establish liaison with cancer centers and individual investigators around the world have stimulated increased communication and cooperation among researchers, as well as more effective utilization of clinical research data.

Proposed Course: Patient data collection will continue through the ICPDES, and efforts are underway to re-establish a European data processing center in addition to the one located in Houston. A new edition of the International Directory of Specialized Cancer Research and Treatment Establishments, published in 1978 and listing 679 such centers located in 80 countries, is in preparation.

Date Contract Initiated: June 1, 1977

Current Annual Level: $255,380

UNIVERSITY OF TEXAS SYSTEM CANCER CENTER (NO1-CO-85405)

Title: Cancer Information Dissemination and Analysis Center (CIDAC) for Cancer Diagnosis and Therapy

Contractor's Project Director: Dr. Eugene McKelvey

Project Officer (NCI): Dr. Dianne E. Tingley

Objectives: The CIDAC provides scientific input necessary to produce information products and services for cancer researchers, and provides guidance to the ICRDB Program, in the area of cancer diagnosis, therapy and rehabilitation.

Major Accomplishments: The CIDAC regularly produces 21 monthly CANCERGRAMs, current awareness bulletins containing abstracts of recently published literature. Ten ONCOLOGY OVERVIEWs, retrospective bibliographies with abstracts concerning high interest topics in clinical cancer research, are published annually. Recently covered topics include glycosyltransferases, radionuclide bone scans, nutritional therapy and thymic factors. The CIDAC performs custom searches of the CANCERLINE data bases in response to requests for information; submits monthly Highlight Reports, pinpointing significant new developments in clinical cancer research; and assists in data base quality control.

Significance to Biomedical Research and Program of the Institute: The CIDAC serves as a valuable resource for the NCI and the worldwide cancer research community in the area of oncology research. The CANCERGRAMS collectively provide comprehensive coverage of this entire field, quickly alerting researchers to new findings with minimal expenditure of effort, and thereby allowing them more time for productive research. ONCOLOGY OVERVIEWs enable researchers to rapidly update their knowledge in emerging areas of research concentration.

Proposed Course: The contractor will continue production of CANCERGRAMs and ONCOLOGY OVERVIEWs and provision of information services.

Date Contract Initiated: June 24, 1976

Current Annual Level: $255,000

SUMMARY REPORT

SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

October 1, 1979 through September 30, 1980

A. Mission

Under the administrative direction of the Director of the Division of Cancer Cause and Prevention serves as the focal point for development of program interests and collaborative efforts (intramural and extramural) in environmental cancer. Overall this involves working with federal and state agencies, industrial and academic institutions, trade unions and consumer groups. Serves as the primary information resource for the Division and the Institute on role of environmental pollution in carcinogenesis. These coordinated efforts fulfill one of the essential functions prescribed in the National Cancer Act of 1971 as amended by the Biomedical Research and Training Amendments of 1978.

Conceptually, this office has taken a holistic approach to assessment of stress from multiple environmental pollutants through collaboration with EPA, NIOSH, OSHA, DOE and FDA. This office represents NCI through presentations at meetings, support of workshops and symposia and publication of proceedings and annals.

The activities of the Interagency Collaborative Group on Environmental Carcinogenesis sponsored by this office and now in its seventh year (50th meeting in March 1980) comprises the collaborative effort with 28 agencies and subagencies. This group is noted for its function as a catalyst for program initiatives and information resource for all these agencies working with NCI. Provides leadership in NCI/EPA and NCI/NIOSH collaborative contractually supported program or environmental and occupational carcinogenesis. The office is a primary interface working with National Toxicology Program in NCI chemical selection for testing for carcinogenesis and reporting to industry and trade associations on results of testing of chemicals for carcinogenicity.

B. Organizational Changes

No major organizational changes occurred other than a staff replacement due to retirement of a senior scientist and addition of staff functions with respect to EPA initiated Environmental Cancer and Heart and Lung Disease Task force required by U. S. Congress.

C. Other Assigned Functions

Operational support to the CSWG (Chemical Selection Working Group) of NCI and the Chemical Selection Subgroup of the Clearinghouse on Environmental Carcinogens (CSSG), the support provided contractually and by staff. The CSSG was terminated in May 1980 so that selection process by NCI will be working directly with NTP. The office maintains a Chemical Selection Planning Group which meets prior to CSWG for planning agendas and prior screening of chemicals.

This office is involved in a number of information contracts, collaborative programs with such organizations as Stanford Research Institute, The International Agency for Research on Cancer (Lyon, France), the Franklin Institute, the Department of Energy, the Mitre Corporation, Brookhaven National Laboratory (DOE) and the large contract collaborative program with EPA and NIOSH referenced above, as well as the Center for Disease Control and the National Library of Medicine.

D. Accomplishments

Interagency Collaboration

The most visible collaboration is through the ICGEC which meets at intervals of 6-8 weeks which held meetings this year on radiation hazard assessment, energy related technologies, and formaldehyde and n-hexane.

In addition, the ICGEC supported a Task Group and initiated, through support from NCI/EPA Collaborative program which is developing a National Data Base on Multi Media Exposure and Body Burden of Environmental Chemicals in Humans. The ICGEC published one proceeding this year on Carcinogenic Hormones (Springer Verlag).

Through the contract project with IARC, the office participated in the preparation of four meetings with three volumes of monographs and a Supplemental Edition on Lists of Carcinogens this year. The office participated in a series of meetings of EPA Task Group on Environmental Cancer and Heart and Lung Disease and provided leadership and chairmanship on subcommittee for Exposure and Metabolism resulting in task group reports essential to this mission.

A meeting of the EPA/HHS Task Force on Environmental Cancer and Heart and Lung Disease - Project Group on Exposure and Metabolic Mechanisms (chaired by Drs. Kraybill and Kelsey) - convened in May resulted in the establishment of a list of topics which will serve as a basis for comprehensive research recommendations and strategies that will be submitted to the Task Force later this year.

Workshops/Collaborative Meetings and Scientific Meetings

This office receives a considerable volume of requests for presentation of papers at scientific meetings and preparation of chapters for books. During the past year the staff members participated in meetings involving papers relevant to water carcinogens (New York, Sao Paulo, Brazil and Colorado Springs, Co.). There is currently involvement with DOE Planning Committee and as Session Chairman for an International Meeting on Risk Assessment for fall of 1980. The primary workshop of significant interest was the May, 1980 Workshop on NCI/EPA and NCI/NIOSH Collaborative Programs on Environmental and Occupational Cancer. Proceedings with papers and abstracts from this workshop were published and an NCI/EPA bound copy of annual report for this collaborative program and projects was distributed.

Interfacing with Trade Associations, Industries, and Trade Unions: This office has continued to be responsive to requests from industry and the trade associations for guidance and asistance in animal model selection, protocol design and development, and exchange of data and information. There are frequent interactions with aproximately 40 trade associations and 200 individual industrial companies, most of which are initiated by the associations and companies as one mechanism to obtain Government guidance for their projected expensive toxicological efforts.

With the aim of eliminating costly duplication and the use of limited, valuable resources, we have attempted to establish international cooperation in the area of chemical testing. Focal points have been identified in the German, Dutch, English and Norwegian industrial communities, and industrial communities in Sweden, Denmark and Canada. Representatives of these communities are routinely supplied with the list of chemicals on, or projected for, test in the carcinogenesis testing program as well as the pre-publication technical reports.

Special Projects on Food Contaminants, Air Pollutants, Water Contaminants, and Drugs and Medical Devices in Environmental Carcinogenesis

From a detailed study on the identification and classification of carcinogens in air and water, working with a contractor, a special listing of carcinogens, mutagens and promoters in these environmental media was prepared with supportive explanation on how classifications were made. During this fiscal year this report will be published and distributed. Additionally, a comprehensive state of the art report on bioassay of water chemicals, epidemiology of water carcinogens and world-wide classification of water pollutants was published through contract support (collaborative project with DOE).

A report was published on food contaminants as carcinogens entitled Tumorigens and Foods: Detection, Regulations and Recent Tumorigenic/Mutagenic Activity Studies. This was a collaborative project with the Franklin Research Center. This compendium of 605 pages deals with major exposure sources, use patterns and human exposure data; a valuable resource on mutagenicity and carcinogenicity of food contaminants.

Cooperative Project of National Cancer Institute and National Toxicology Program on assembling a list of chemicals for testing and research as required by U. S. Congress under the Biomedical Research and training Amendments of 1978. In response to paragraph 4D of the amendments sections I and II was another cooperative project. This listing of over 300 chemicals was facilitated through work of CSWG and NTP Chemical Nomination and Selection Committee.

Preparation and production of the Second Biennial Report to Congress on Research Activities of Relevance to the Clean Air Act was accomplished as it relates to UV radiation effects. Major areas encompassed were epidemiologic studies which revealed that sunlight exposure correlated with increased incidence of non-melanoma skin cancer and the UV radiation enabled photochemical reactions between DNA and other substances.

A review of proposals submitted in response to an RFP for a data base concerned with the presence of carcinogens in drugs and medical procedures has been completed, and an award will be made shortly. An RFP dealing with information on carcinogens in cosmetics has been prepared and will be released within the fiscal year.

Committees and Subcommittees

The staff of this office served on various Task Groups, Committees and Subcommittees which include the following: Task Group EPA/HHS on Environmental Cancer and Heart and Lung Disease and Subcommittee of Task Group on Exposure and Metabolism; EPA Interagency Regulatory Liaison Group - Cadmium Working Group; EPA Research Relating to Ozone Protection, Interagency Coordinating Group; subcommittee on Stratospheric Pollution of Committee on International Environmental Affairs (DOS); CEQ Environmental Data and Monitoring Work Group, Water and Data Monitoring; Interagency Collaborative Group on Environmental Carcinogenesis; Chemical Selection Working Group of NCI; NTP Chemical Selection and Nominating Committee; Toxicology Information Subcommittee Task Group for the Laboratory Animal Data Bank; Committee on Food Irradiation, General Committee on DOD Food Program; Interagency Working Group on Nitrite Research; Committee for the PL-480 project on Nephrotoxic Chemicals (FDA); Steering Committee for Water Chlorination: Environmental Impact and Health Effects; DOE Planning Committee for Symposium on Health Risk Analysis.

Lectures and Publications

Members of the staff of this office have delivered lectures and presented papers to 8 professional organizations during the year. In addition the following papers and books were published.

Kraybill, H. F.: Foreword. In Lingeman, C. H. (Ed.): Recent Results in Cancer Research - Carcinogenic Hormones. Proceedings of the 24th and 25th Meetings of the Interagency Collaborative Group on Environmental Carcinogenesis. New York, Springer Verlag, 1979.

Kraybill, H. F.: By Appropriate Methods: The Delaney Clause. In Coulston, F. (Ed.): Regulatory Aspects of Carcinogenesis and Food Additives, The Delaney Clause. New York, Academic Press, 1979, pp. 61-83.

Kraybill, H. F.: Evaluation of public health aspects of carcinogenic/mutagenic biorefractories in drinking water. Preventive Medicine, 9: 212-218, 1980.

Kelsey, M. I.: Tests for mutagenicity. Symposium on Bacterial Metabolites in the Human Intestine: Physiological and Pathophysiological Role, American Society for Microbiology, 80th Annual Meeting, Miami Beach, Florida, May, 1980.

Highlights

There were several significant developments and program improvements this year. The supervision, management and performance in the conduct of the NCI/EPA and NCI/NIOSH collaborative research programs were notable. This improvement was evident in the publication of an annual report for the NCI/EPA research program in the area of environmental cancer. This represented an initiative on the part of the staff. Also, a successful workshop was held on both the NCI/EPA and NCI/NIOSH collaborative projects.

This year we were able to achieve the development of important data bases on water carcinogens, air carcinogens and diet and food carcinogens. The reports on this data will have wide utilization for chemical selection process, for guidance in interagency programs and development of position papers.

The work of the Chemical Selection Working Group has been well-systematized and organized into the production of class studies (use and structure) which have wide utility. They will be published this year in the open literature. The work of this group has been an important resource and contribution to the development of selection and nomination of chemicals from NCI to the National Toxicology Programs (NTP). NTP has become quite dependent on this resourse.

Another important accomplishment of this office during the year through some assistance by a contractor was the development of a list of 343 chemicals that were specified for carcinogenicity testing and or research as required by NCI, NIOSH, NIEHS, FDA, USDA, DOD and other agencies through our interagency network. The submission of this list was in response to recent legislation enacted by the U. S. Congress (the so-called Maguire Amendment) under the Biomedical Research and Training Amendments of 1978. This listing to be submitted to the Department and then to the U. S. Congress indicates how the Department responded or prioritized these chemicals for testing and research.

CONTRACT NARRATIVES

OFFICE OF SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

Contract	Title	Page No.
Franklin Inst. Research Labs. (NO1-CP-85644)	Survey of Compounds Which Have Been Tested for Carcinogenicity, 1978 Supplement	90
Stanford Research Inst., Int. (NO1-CP-95607)	Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Cause and Prevention, National Cancer Institute and to provide Chemical Process Production and Economic Information as support to the International Agency for Research on Cancer (IARC)	90
Energy, Dept. of (YO1-CP-80202)	Study of the Human Health Significance of the Potentially Carcinogenic Energy Related Organic and Inorganic Contaminants in Water	91
Energy, Dept. of (YO1-CP-90202)	To Establish an Office to Coordinate Information and Technological Transfer in Environmental Mutagenesis and Carcinogenesis	92
U.S. Environmental Protection Agency (YO1-CP-80205)	Interagency Agreement for Performance of Collaborative Studies in Area of Environmental Cancer, NCI/EPA	93
Int. Agency for Research on Cancer (NO1-CP-45608)	Evaluation of Carcinogenic Risk of Chemicals to Man	94
Battelle Memorial Institute (LM5-4747)	Development and Implementation of Laboratory Animal Data Base	95
Center for Disease Control (YO1-CP-60215)	Human Health Consequences of Polybrominated Biphenyls (PBBs) Contamination of Farms in Michigan	95
National Inst. of Occupational Safety and Health (YO1-CP-60505)	Conduct of Research on Occupational Carcinogenesis	96
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FRANKLIN INSTITUTE RESEARCH LABORATORIES (NO1-CP-85644)

Title: Survey of Compounds Which Have Been Tested for Carcinogenicity, 1978 Supplement

Contractor's Project Director: Dr. Bruce Kleinstein

Project Officer (NCI): Dr. H. F. Kraybill

Objectives: This project provides a resource for preparation of PHS-149, Survey of Compounds for Carcinogenicity for the Year 1978 - literature survey.

Major Findings: There was a lapse from 1973-1978 in preparation of this reference source used nationally and internationally. The publication will be available in fall of 1980 and distribution will be made for program managers as a ready reference source.

Significance to Biomedical Research and Program of the Institute: This project will provide the information resource for staff at NCI, academia and other research organizations setting forth data gleaned from the 1978 worldwide literature on bioassay results on chemicals tested in animals for carcinogenicity. The report will be a standard reference source for those responding to all types of inquiries and for those engaged in testing programs.

Proposed Course: The contractor has completed the survey for 1978 which is currently being printed by the Government Printing Office. Evaluation of this survey ascertained desirability for continuance with a 1974-1977 literature search and a survey of 1979 and 1980. RFP is being processed at this time.

Date Contract Initated: September 30, 1978

Current Annual Level: $110,000

STANFORD RESEARCH INSTITUTE, INTERNATIONAL (NO1-CP-95607)

Title: Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Cause and Prevention, National Cancer Institute and to provide Chemical Process Production and Economic Information as Support to the International Agency for Research on Cancer (IARC)

Contractor's Project Director(s): Dr. Tucker Helmes, Ms. Mary Doeltz

Project Officer (NCI): Dr. H. F. Kraybill

Objectives: This project is an important information and data resource. The project has five tasks as follows: 1) Support to the Chemical Selection Working Group; 2) Support to International Agency for Research on Cancer; 3) Updating and expanding the MARK II Data Base; 4) Data Processing Services and Support; 5) Overall Support to Carcinogenesis Research Program. In addition, there may be a task added to give support to the National Library of Medicine.

Major Findings: This current project is in the first year of a planned five-year project. It is an invaluable information and data resource for not only NCI but many other federal agencies and, of course, the IARC in Lyon, France. Because of SRI's unique data base on production and exposure of some 30,000 industrial chemicals it has facilitated the systematic selection of chemicals for research and testing in chemical carcinogenesis. The International Agency for Research has depended on SRI for certain portions of their monographs on Evaluation of Carcinogenicity of Chemicals. SRI has completed 9 class studies as follows: Metals, Phase III; Biocides; Biological Intermediates; Fungicides and Nematocides; Anhydrides; Alkaloids; Thiazoles; Alkythiocyanates; Arylsulfonamides; Vol. 19 of the IARC monographs was reviewed to select chemicals for nomination to the NTP.

Proposed Course: SRI was the successful bidder for continuance of this effort and a new contract has been signed for a 5-year period. The contractor will continue its support of the NCI nominations of chemicals for testing to the National Toxicology Program. More emphasis will be given to position papers and special information projects in support of the work of the Scientific Coordinator for Environmental Cancer.

Significance to Biomedical Research and Program of the Institute: This project has continued to be a most significant resource to research and program staff with respect to management of program in the area of environmental cancer, carcinogenesis projects and testing program relevant to mechanisms for selection of chemicals. The dossiers and/or summary sheets prepared under this project have been useful to NCI, NIEHS, FDA, NIOSH, CPSC, and OSHA as authorative reference documents on chemicals based on a repository of 30,000 chemicals.

Date Contract Initiated: Nov. 1, 1979

Current Annual Level: $969,000

DEPARTMENT OF ENERGY, ENVIRONMENTAL PROGRAMS, DIVISION OF BIOMEDICAL AND ENVIRONMENTAL RESEARCH (Subcontractor, Mitre Corporation)(Y01-CP-80202)

Title: Study of the Human Health Significance of the Potentially Carcinogenic Energy Related Organic and Inorganic Contaminants in Water

Contractor's Project Officer (Mitre): Ms. Judith Hushon

Project Officer (NCI): Dr. H. F. Kraybill

Project Officer (DOE): Dr. Nathaniel Barr

Objectives: To develop a state of the art report on bioassay, monitoring, identification, and classification of energy and environmental chemicals that are carcinogens and mutagens, and delineation of an evaluation of epidemiological studies that relate such aquatic contaminants to adverse effects in aquatic and mammalian species, including carcinogenic effects from such exposures to water contaminants.

Major Findings: This project which has been completed, has delineated all the bioassays used in various animal models, provided a description of all monitoring systems in all federal agencies, a description and cataloguing of over 2,300 chemicals in raw water and specific listing of carcinogens and mutagens in drinking water and an indepth evaluation of the dozen or more epidemiological studies on populations exposed to these contaminants.

Significance to Biomedical Research and Program of the Institute: The preliminary reports made available through a briefing session in July 1979 indicates that this comprehensive survey will provide a very significant report (probably 3 volumes) which puts in perspective for the biomedical community and specifically government agencies, including DOE and NCI, a clear picture of the impact of these contaminants on environmental health including cancer.

Proposed Course: This project was extended beyond the original one-year plan to eighteen months. It has been completed and the reports are currently being printed.

Date Contract Initiated: March 30, 1978

Current Annual Level: $115,999

DEPARTMENT OF ENERGY (Subcontractor - Associated Universities, Brookhaven National Laboratory)(YO1-CP-90202)

Title: To Establish an Office to Coordinate Information and Technological Transfer in Environmental Mutagenesis and Carcinogenesis

Contractor's Project Officer: Dr. Alexander Hollaender

Project Officer (NCI): Dr. H. F. Kraybill

Objective: The National Cancer Institute in collaboration with EPA, NIEHS and DOE will support training centers for instruction of national and international scientists through leadership of Dr. Hollaender on procedures and information and data bases in the area of mutagenesis and carcinogenesis.

Major Findings: Several workshops have been held to provide such orientation at several academic institutions in this country and abroad. In providing sorely needed skills for the laboratory worker the project has achieved a cadre of trained experts which can interpret genetic/toxicology tests which is important in assessment of health aspects and impact of mutagens and carcinogens.

Significance to Biomedical Research and Program of the Institute: Training is an integral part of providing properly qualified scientific investigators who have the required skills as laboratory workers. Additionally, it provides a cadre of future program managers and science administrators who are capable of making biomedical evaluations and decisions requisite in research progrms of the scientific community at large as well as NCI.

Proposed Course: This training effort is expected on a collaborative basis with other agencies to extend from 1979 through 1981 at which time an evaluation will be made as to the success of this program.

Date Contract Initiated: April 1, 1979

Current Annual Level: $53,700

U.S. ENVIRONMENTAL PROTECTION AGENCY (Office of Toxic Substances and Office of Research and Development) (YO1-CP-80205)

Title: Interagency Agreement for Performance of Collaborative Studies in Area of Environmental Cancer, NCI/EPA

Contractor's Coordinators or Project Directors: NCI, Dr. H. F. Kraybill; EPA/OTS, Dr. C. C. Lee; EPT/RD, Dr. Wayne Galbraith

Objective: In response to request from the Congress and OMB, NCI entered into a collaborative arrangement with EPA to conduct experimental and epidemiological studies in the area of environmental cancer. This program initiated two years ago has now matured into 30 projects where NCI staff and EPA staff serve as project and coproject officers on studies dealing with environmental exposures and relevance to cancer (see attached table of projects).

Major Findings: One project in this collaborative effort has resulted in the publication of a report entitled "Chemicals Found in Human Biological Media: A Data Base. This report reflects exposures to food, air and water contaminants, as well as pharmaceuticals. This data base will provide a valuable resource to regulatory agencies, research agencies, and other concerned federal and private agencies.

Significance to Biomedical Research and Program of the Institute: This collaborative program has achieved greater cognizance on the part of NCI on real problems of environmental exposures. The projects are oriented toward problems dealing with exposures to water contaminants, air pollutants, chemicals on which ultimate standards are developed to protect the public. Some penetrating studies on such carcinogens as chloroform, aromatic dyes, asbestos, UV radiation are practical concerns that are being addressed. The work performed is most responsive to public health needs. The collaborative program is being followed with much interest by the Congress.

Proposed Course: Many of the projects are planned for 3 to 5 years which should provide an important data base and information useful to NCI and EPA. More experimental studies will be introduced as companion projects to the epidemiological projects. The funding will be maintained at current level with an equal split between NCI and EPA.

Date Contract Initiated: June 22, 1978

Current Annual Level: $4 million

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (NO1-CP-45608)

Title: Evaluation of Carcinogenic Risk of Chemicals to Man

Contractor's Project Director: Dr. John Higginson

Project Officer (NCI): Dr. H. F. Kraybill

Objective: The IARC prepares monographs on environmental chemicals relevant to evaluation of carcinogenic risk of chemicals to man. Implementation of the IARC Monograph program involves three steps: 1) the collection of data relevant to the assessment of the carcinogenic risk of chemicals to which humans are exposed; 2) the critical analysis and evaluation of these data by international working groups of experts in chemical carcinogenesis and related disciplines; and 3) the publication and dissemination of these data as IARC Monographs. NCI receives approximately 200 copies for distribution to NCI staff and other interested program staff of 28 federal agencies.

Major Findings: The 22 Monographs published to date have become an authoritative international reference source. These lucid and concise reports provide a ready reference on evaluation of carcinogenicity and risk of important chemicals through an appraisement of animal and human data.

Significance to Biomedical Research and Program of the Institute: As an information and data resource, the IARC Monographs have become an indispensable authoritative reference to provide an instantaneous judgement as to the appraisement of carcinogenicity of a wide spectrum of chemicals both by structure and use class. Without this reference source, progress of NCI and federal regulatory programs in the USA would be several handicapped.

Proposed Course: This contractor will continue as in the past years to convene 3-4 advisory committee meetings, or workshops, per year to evaluate the literature and give guidance to IARC staff in preparing and editing material for the monographs.

Date Contract Initiated: May 15, 1974

Current Annual Level: $387,206

BATTELLE MEMORIAL INSTITUTE (LM5-4747)

Title: Development and Implementation of Laboratory Animal Data Base

Contractor's Project Director: Mr. Richard C. Simon

Project Officer (NCI): Dr. Thomas P. Cameron

Objective: This activity is designed to provide to the biomedical research community a major tool to lessen the cost of animal studies, minimize the numbers of animals utilized, and yet increase the validity of these experiments.

Major Findings: A computerized, on-line interactive bank of control animal, base-line data has been refined through a series of reprogrammings until it now is adaptable to the needs of both the neophyte researcher as well as those highly experienced in searching computer banks. The present capacity is some 800,000 individual observations on 25,000 animals. Midway through this fiscal year the Laboratory Animal Data Bank was made available to the public user across the country. It will not be possible to assess the exact quantity of use until a year's experience has been gained, but initial indications are that the system has received an enthusiastic response.

Significance to Biomedical Research and Program of the Institute: This system will allow researchers to more effectively plan and evaluate their studies. Considerable savings will result in animals, funds, equipment, space and people with a concurrent increase in scientific validity. Species and strains, e.g., non-human primates, can be studied whose limited availability would otherwise make it impractical to consider them as research subjects.

Proposed Course: Computer programs will be modified and added to as experience dictates especially in the areas of statistics and pathology retrieval. The major effort will be the acquisition, validating and processing into the bank of large amounts of biological information. This bank enhancement, and the effort necessary to respond to the just initiated public utilization of the system, will be primary thrusts. The 4 years of preparation for this phase will be carefully evaluated in the following year.

Date Contract Initiated: June 30, 1975

Current Annual Level: $350,000

CENTER FOR DISEASE CONTROL (YO1-CP-60215)

Title: Human Health Consequences of Polybrominated Biphenyls (PBBs) Contamination of Farms in Michigan

Contractor's Project Director: Dr. Clark W. Heath, Jr.

Project Officer (NCI): Dr. Thomas P. Cameron

Objective: As a consequence of the distribution of animal feeds contaminated with PBB, the inhabitants of at least 412 Michigan farms as well as a wide surrounding belt of consumers, were accidently exposed to that potentially threatening compound. This activity defines and recruits large cohorts of individuals (of several degrees of exposure) and will engage in a long-term follow-up primarily with respect to the incidence of cancer.

Major Findings: The major effort to date has been the development of cohorts. Two blood samples have been taken from all PBB exposed individuals and analyzed for this compound as well as polychlorinated biphenyls (PCBs) which is of equal interest scientifically and provides gratuitous information. Certain high risk groups (high blood level individuals engaged in the production of the PBB compound and children born during or after the incident)

continue to be monitored closely. The initial scientific impression that there were little or no immediate or acute effects on the subject population appears to have been verified.

Significance to Biomedical Research and Program of the Institute: This is a unique situation in which a large group of people had a known exposure to a potential carcinogen under sharply defined circumstances of geography and time. Unfortunate subsequent rises in cancer incidence, if they occur, can thus be charted in this prospective study and correlated with modes and amount of exposure. Major scientific observations on this particular compound and structurally allied compounds can be developed.

Proposed Course: Cohorts having been defined and recruited, the major effort for the foreseeable future is to maintain their integrity for the long-term determinations. Deaths, illness, pregnancies, etc., will be carefully monitored. It is anticipated that the scaling down of effort will continue to some degree because of the completion of major tasks.

Date Contract Initiated: April 8, 1976

Current Annual Level: $200,255

NATIONAL INSTITUTE OF OCCUPATIONAL SAFETY AND HEALTH (YO1-CP-60505)

Title: Conduct of Research on Occupational Carcinogenesis

Contractor's Project Director: Dr. Kenneth Bridbord

Project Officer (NCI): Dr. Thomas P. Cameron

Objective: This agreement was originated as a Congressionally mandated effort to integrate and thereby enhance the activities of both the National Cancer Institute and the National Institute for Occupational Safety and Health in investigating chemical related occupational cancer.

Major Findings: To date some 71 distinct projects have been initiated, of which approximately 50 are in progress. Most of the projects were reported on in a combined NCI/EPA/NIOSH Collaborative Workshop held in April, 1980.

Significance to Biomedical Research and Program of the Institute: The merging of the unique capabilities of each organization into a concerted investigation of a variety of industrial situations promises an acceleration of the response to the question of the quantification of occupationally-caused cancer. In that quest, answers to the many questions concerning acute toxicity, safe ambient levels, etc., which are more germane to the NIOSH mission, will accrue. NIOSH contributes the capability of freely entering industrial operations as well as considerable experience in surveying potentially dangerous situations therein.

Proposed Course: The on-going projects shall be continued as dictated by their objectives and workscopes. New projects shall be begun as a result of initiation by the staff of either organization and relevance and technical review by a combined ad hoc committee of the senior staff members of both institutes. Obligation of funds by the NCI for the ensuing fiscal year shall in no case exceed the sum of four million dollars.

Date Contract Initiated: September 23, 1974

Current Annual Level: $4 million

BASIC ORDERING AGREEMENT (NO1-CP-95630)

Title: Information Support Contract on Environmental Agents

Contractors and Respective Project Directors:

SRI, International: Dr. Tucker Helmes, Mary Doeltz, Caroline Sigman

Syracuse Research Corp.: Dr. J. Santodonato, Clyde Beigh

Science Applications, Inc.: Dr. Jack Tobler, Michael Pasqua

Tracor Jitco: David Lindsell, Dr. Harold Seifried, Dr. G. R. Pielmeier

Project Officers (NCI): Dr. Morris Kelsey and Dr. H. F. Kraybill

Objectives: This project provides a resource for preparation of state-of-the-art and assessment studies for the use of the staff.

Major Findings: None to date

Significance to Biomedical Research and Program of the Institute: This project will provide an information resource for the staff at NCI, and through interagency collaborative efforts to the rest of NIH, other federal and state agencies, industrial and academic institutions, trade unions and consumer groups. As proposed, studies to be performed under this contract include state-of-the-art reports on selected air and food contaminants, assessment of carcinogenic and mutagenic hazards in various media, and comparison studies of various carcinogen testing systems and animal models.

Proposed Course: The project was designed to make awards over the period of a year for the completion of small task orders, each awarded individually. The project is to be renewed for a period of one year.

Date Contract Initiated: March 1, 1980

Current Annual Level: $300,000

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04548-08 OD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Registry of Experimental Cancers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Harold L. Stewart	NIH Scientist Emeritus	DCCP	NCI
OTHER:	Thelma B. Dunn (until 4/1/80)	Consultant	DCCP	NCI
	Margaret K. Deringer (Margaret D. Miller)	Biologist	DCCP	NCI
	Morton H. Levitt (until 7/1/80)	Medical Pathologist	DCCP	NCI
	Bernard Sass	Veterinary Medical Officer	DCCP	NCI
	Cornelia Hoch-Ligeti	Guest Worker	DCCP	NCI
	Carel F. Hollander	Consultant	DCCP	NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Division of Cancer Cause & Prevention, Office of Director

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The objectives of the Registry of Experimental Cancers are 1) the storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primary rodents) and 2) the use of such information for research and educational purposes.
2. The Registry has acquired a total of 1883 (76 since the 1979 report) single or group accessions from investigators outside the NCI. Approximately 53,200 (3,200 since the 1979 report) records have been prepared for coding and coded.
3. Thirty-two investigators have come to the Registry for study and consultation on single or multiple visits. Two foreign investigators were guests at the Registry for 2 days and 3 weeks respectively.

Project Description

Objectives: 1) The storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents), and 2) the use of such for research and educational purposes.

Methods Employed: The methods employed in the work of the Registry involve the selection of protocols, pathologic material, including histologic slides, paraffin blocks, and illustrations in the form of lantern slides, gross photographs, and photomicrographs in black and white and in color. The work of the Registry also includes the collection of records of experiments, reprints and references on this material. The Registry of Experimental Cancers possesses a large collection of spontaneous and induced cancers and other lesions. The pertinent information on the collection is indexed. Many of the data have been prepared for and entered into the computer. The Registry accesses material from investigators at NCI, other institutes of NIH, other governmental agencies, industrial laboratories, and universities here and abroad. A total of 1883 single or group accessions from investigators outside of NIH have been processed since 1971. The Registry has prepared and is preparing Study Sets of slides, with explanatory notes, relating to particular cancers of rodents.

The Registry has Study Sets of slides on "Hematopoietic and Lymphoreticular Neoplasms", "Induced and Spontaneous Tumors of Small Intestine and Peritoneum in Mice", "Tumors and Nonneoplastic Proliferative Lesions of the Lungs of Mice", "Induced Tumors of the Liver in Rats", and "Mammary Tumors in Mice" which are loaned,with descriptive material, to investigators who request them. Twenty-three loans up to two months have been made this year.

Investigators come to the Registry for study and consultation. There have been single or multiple consultations with 32 individuals since the 1979 report. Two foreign investigators have been guests at the Registry for 2 days and 3 weeks respectively.

Pathology of Tumours in Laboratory Animals, Vol. II Tumours of the Mouse has been published. Five of the 25 chapters were written by members of the Registry's staff. Material from the Registry was supplied to the authors of eight other chapters.

Major Findings: The functions (outlined in objectives) of the Registry in the field of cancer research are such that there will be no major findings to report.

Significance to Biomedical Research and the Program of the Institute: The availability of the wealth of material possessed by the Registry advances the knowledge of spontaneous and induced disease processes in animals. It is a national and international resource.

The existence of the Registry will contribute to the standardization of nomenclature of cancers and other lesions in laboratory rodents. Slides and protocols from the Registry are used to illustrate and describe lesions discussed at the weekly slide conferences.

The members of the Registry serve as consultants in the monitoring of pathology from laboratories of NCI, of other institutes of NIH and elsewhere.

The Subcommittee on Rat Liver Tumors, appointed by the Institute of Laboratory Animal Resources, NRC, NAS carried out its work at the Registry of Experimental Cancers where it met one or two days each month over a period of 18 months. The members of the Subcommittee studied the pathologic material in the Registry's Study Set on Rat Liver Tumors and other pathologic materials accessed at the Registry. The publication of the report entitled "Histologic Typing of Liver Tumors of the Rat" appeared in January of this year (J Natl Cancer Inst 64:177-206, 1980). During the period from the date of publication until April 30, 1980, the Registry has received 2,116 requests for reprints. This histologic classification and typing of rat liver tumors is calculated to promote uniformity of diagnoses from one laboratory to another in this country.

The Director General of the World Health Organization designated the Registry of Experimental Cancers as the WHO Collaborating Centre for Reference on Tumours of Laboratory Animals on 26 October 1976. This is the only such registry in the world to be so designated by the WHO. This will expand communications between scientists of this country and those of other countries, now numbering 153, which are members of WHO.

Proposed Course of the Project: The Registry will continue and expand all of its activites (already set forth in this report).

Publications

Dunn, T.B.: Tumours of the parathyroid gland. In Turusov, V.S. (Ed.): Pathology of Tumours in Laboratory Animals. Vol. II Tumours of the Mouse. Lyon, IARC, 1979, pp. 469-474.

Dunn, T.B.: Tumours of the adrenal gland. In Turusov, V.S. (Ed.): Pathology of Tumours in Laboratory Animals. Vol. II Tumours of the Mouse. Lyon, IARC, 1979, pp. 475-486.

Munoz, N., Dunn, T.B. and Turusov, V.S.: Tumours of the vagina and uterus. In Turusov, V.S. (Ed.): Pathology of Tumours in Laboratory Animals. Vol II Tumours of the Mouse. Lyon, IARC, 1979, pp. 359-384.

Stewart, H.L.: Tumours of the soft tissues. In Turusov, V.S. (Ed.): Pathology of Tumours in Laboratory Animals. Vol. II Tumours of the Mouse. Lyon, IARC, 1979, pp. 487-526.

Stewart, H.L., Dunn, T.B., Snell, K.C. and Deringer, M.K.: Tumours of the respiratory tract. In Turusov, V.S. (Ed.): Pathology of Tumours in Laboratory Animals. Vol. II Tumours of the Mouse. Lyon, IARC, 1979, pp. 251-288.

Sass, B.: The occurrence of a bilateral mandibular mast cell neoplasm in a mouse with lymphocytic leukemia. Lab. Anim. Sci. 29:492-494, 1979.

CARCINOGENESIS INTRAMURAL PROGRAM

SUMMARY REPORT

LABORATORY OF BIOLOGY

October 1, 1979 through September 30, 1980

"Plans, develops, and conducts a research program aimed at (1) elucidation of the role of chemical, physical and biological agents in the process of carcinogenesis (2) characterization of cellular alterations associated with carcinogenesis, (3) evaluation of the relationships between mutagenesis and carcinogenesis, and (4) the development and definition of in vitro cellular transformation systems for the rapid detection of the carcinogenic actions of chemicals."

The Laboratory of Biology has established an interdisciplinary program in carcinogenesis emphasizing a biological approach in tumor biology and somatic cell genetics. The long range goal is to understand the mechanism of neoplastic transformation through the use of cell biology, cytogenetics, DNA metabolism, and immunology. The study of chemical carcinogenesis has been approached in vitro in terms of target cell insult and nontarget cell interactions. In vitro cell culture models have permitted the study of factors which influence mammalian cell transformation, a primary step in neoplasia, as well as the study of environmental agents that are potential carcinogens.

Thus far, the laboratory has reported carcinogen-induced neoplastic transformation of embryo or fetal derived cells of rat, guinea pig, human, and Syrian hamster. In addition, chemicals have been utilized to demonstrate transformation with a mouse cell line, Balb/3T3. We have evidence that Chinese hamster embryo cells may also be transformed by chemical carcinogens. In models in which cells are first seeded for colony or focus formation, a dose response relationship within certain limits exists between the chemical carcinogen concentration and the number of cells that are transformed. The distribution of the transformations exhibits a Poission distribution and the incidence of transformation in vitro correlates with in vivo activity of the chemical carcinogen. The relevancy of transformation to cancer is that the transformations can be isolated and produce tumors when injected into appropriate hosts. Thus, evidence exists that chemically induced carcinogenesis can be studied in vitro.

Mammalian fibroblast cells in vitro for the study of transformation has contributed to our knowledge of chemical carcinogenesis. The molecular events involved in chemical carcinogenesis would not be expected to depend on the specific cell type. In fact, the required steps in chemical carcinogenesis, uptake, metabolism and covalent binding to DNA, appear to be the same in epithelial and fibroblast cells. Nevertheless, the study of epithelial cell transformation may offer several distinct advantages not found in established fibroblast systems. Specifically, epithelial cells which express a differentiated function may also manifest biological markers specific for neoplastic transformation. Moreover, in vivo studies have shown that the induction and

subsequent growth of adenocarcinomas in several target cells are dependent on circulating levels of protein and steroid hormones. Therefore, our laboratory has broadened the study of cell transformation to include epithelial cells derived from breast tissues. A development of an in vitro transformation system for epithelial cells would provide the initial opportunity for the study of hormonal modulation of cancer. Preliminary studies are focused on the selection of a target cell to study epithelial transformation. Because of their high susceptibility to carcinogenic polycyclic hydrocarbons, the epithelial cells of the rat mammary gland provide an excellent model for the study of in vitro transformation of a differentiated cell. Preliminary studies have established that mammary epithelial cells grown in vitro possess the enzymatic mechanism for the metabolism of carcinogenic hydrocarbons. Furthermore, after in vitro carcinogen treatment, the mammary epithelial cells express anchorage independent growth, a phenotypic change often correlated with the ability to form tumors in vivo. The studies provide an essential framework for the investigation of epithelial transformation in vitro. Additional research, using innovative techniques, will be directed to the processes of differentiation and carcinogenesis.

A fundamental question is whether a carcinogenic chemical transforms or selects pre-existing transformed cells, possibly by the elimination of some normal cells by the toxic effect of the carcinogenic insult. With the Syrian hamster embryo system, a dose-response relationship and a zero-threshold level is observed. The transformation frequency, when plotted relative to carcinogen concentration results in a curve with slope of approximately one on a log-log basis. Thus, transformation is consistent with the one-hit hypothesis. Appropriate statistical procedures for fitting one-hit curves based on the assumption that each experimental unit has an independent and equal probability of being positive, have been applied to data obtained by chemical carcinogen alone or after different treatments. Under the assumption of binomial variation, the one-hit curves have been shown to fit several sets of experimental data. Maximum likelihood estimation methods have been used with two statistical tests to fit. Enhancement of the effect of irradiation on chemical transformation was quantified by the ratios of the parameters of the one-hit curves, and standard errors of this measure were developed. Statistical examination of randomness was applied to the distribution of the number of transformed cells among the dishes. In addition, the total number of cells at risk was estimated with statistical methods developed for use with the number of dishes in an experiment. The biological and statistical analyses strongly support the conclusion that transformation is an inductive phenomenon.

In studying some of the factors that influence the incidence of transformation, we were interested in determining the extent to which one agent influences the frequency of transformation by another agent from a different category of carcinogens. The modulation of transformation frequency as a result of exposure to multiple agents, carcinogens and noncarcinogens, is important to understanding the mechanisms of transformation and because combinations of various agents may be responsible for increased risk of human cancer. One observation is that pretreatment of cells by X-irradiation or by an alkylating agent such as methylmethane sulfonate (MMS) results in an increase in the number of transformations by a variety of carcinogens. With these conditions, X-irradiation

did not produce any transformation whereas MMS did produce rare transformations. When cells in mass culture were pretreated with X-irradiation or with MMS for 1 hr before being plated for colony formation, and then treated with benzo(a)pyrene (BP), dimethylbenz(a)anthracene (DMBA), 3-methylcholanthrene (MCA), acetoxyacetylaminofluorene (AcAAF), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) at 48 hrs, significant enhancement was obtained with all the carcinogens used. Furthermore, if the chemical concentration was doubled, the enhancement ratio remained constant. The ratio of enhancement with MMS pretreatment ranged from a low of 4 with MNNG to a high of 7.4 with BP; however, after pretreatment with radiation, the enhancement factor was greater than that obtained with MMS pretreatment with a low of 7.5 for MNNG and a high of 8.8 for DMBA. If ethylmethane sulfonate was substituted for MMS and BP added after 48 hrs, the enhancement (7.5) was similar to that obtained with MMS. The enhancement of transformation by X-ray or alkylating agents is independent of whether the chemical carcinogen used is an alkylating agent, uv-like compound or a polycyclic hydrocarbon.

The effect of irradiation was further probed by substituting uv irradiation (254 nm) for X-irradiation. Syrian hamster cell strains are especially sensitive to uv, as indicated by colony-forming ability (D_{37}=58 erg/mm^2). A typical survival curve measuring post-irradiation growth had a small shoulder. The lethality observed with these cells is similar to that reported for a number of cell lines and to that obtained by us using normal 1220 human fibroblast cells. In the latter case, the uv dose corresponding to 37% survival of colony-forming ability was about 67 ergs/mm^2. UV irradiation increases transformation in proportion to doses in the range of 7.5 to 61 ergs/mm^2. When cells seeded to form colonies were treated with uv irradiation followed by BP or AcAAF at various times from 6 min to 72 hr after uv irradiation, the incidence of transformation was additive or the same as that obtained with chemical alone. The combination of a constant amount of X-irradiation, 250R, followed by either 15 or 30 ergs/mm^2 of uv irradiation 48 hr later, resulted in a significant enhancement of transformation over that obtained by uv irradiation alone. The results with X-ray and uv irradiation indicated that the enhancement of transformation followed an X-ray type of insult. It has been postulated that transformation is the result of a gene mutation. The uv transformation frequency of 10^{-3}/ergs/mm^2 is 10^2-10^4-fold greater than the reported uv mutation frequency with mammalian cells. This difference suggests that the target for transformation is significantly larger than that for a single locus mutation. The exact cause of the transformation enhancement resulting from the interaction of these agents is unknown, but the enhancement associated with pretreatment cannot be related to partial cell synchronization or disruption in the cell cycle. Furthermore, hamster cells pretreated with 250 μM MMS demonstrated no alteration in normal cell DNA synthesis through 48 hr post-treatment. Similar results are obtained with X-irradiation. Therefore, the relationship between pretreatment of cells with X-ray or MMS and the cells ability to repair uv-induced DNA damage was also investigated. UV was chosen because repair of DNA damage caused by uv irradiation (254 nm) is easily quantitated. An endonuclease sensitive site assay was used to measure the rate of pyrimidine dimer excision under enhancement and control conditions. Independent of the treatment conditions, only 25 and 35% of the pyrimidine dimers are excised by 24 and 72 hr post-uv, respectively. However, the presence of residual pyrimidine dimers does not prevent semiconservative DNA

synthesis as more than 70% of the genome was replicated within 24 hr in uv - irradiated cells. The rate of post-replication repair is functionally defined as the time required for small pulse labeled DNA to be chased into parental size DNA in uv-irradiated cells. Post-replication repair was measured in cells pretreated 48 or 72 hr with 250R of X-ray and compared to the rate in cells treated with uv alone. The size of pulse labeled DNA nascent strands was the same as indicated by their rate of chase into parental length DNA whether or not the cells had been treated with X-ray (48 or 72 hr) prior to uv. Moreover, the size of pulse labeled nascent strands is smaller in uv irradiated than in non-uv-irradiated cells under all pre-treatment conditions. Therefore, the enhancement of transformation by X-ray does not result from a change in the rate of pyrimidine dimers excision or post-replication repair. The results suggest that other repair systems, such as the recovery of nascent strands to normal size, may be important to cell survival and to the mechanism of transformation and its enhancement.

Another fundamental aspect of our program has been to determine the cytogenetic alterations responsible for or associated with the conversion of normal cells into malignant cells. The role of chromosomal abnormalities in neoplastic transformation has been elaborated by utilizing cells transformed by chemical carcinogens or viruses and comparing these to the tumors that they produce in vivo and to normal cells. The presence of chromosomal abnormalities in malignant cells has been widely recognized; however, the significance of these alterations has been the subject of much debate. Studies based on a number of independently derived Syrian hamster cell lines transformed with the same or different chemical carcinogen or virus have resulted in the conclusion that different carcinogens result in transformation in which no chromosome change in terms of chromosome balance occurs. On the other hand, different carcinogens may produce transformation associated with a specific abnormality but no universal structural or numerical alteration was related to a specific chemical or virus transformation. Therefore, the chromosome changes were independent of the etiologic agent. When viral transformed cells were used and in situ hybridization of the DNA virus and the complementary RNA done, a lack of structural changes in the chromosome extending to the general localization of the transforming virus or its components on the chromosomes of transformed cells was found.

Significant contributions towards understanding the initial carcinogen induced chromosomal damage during carcinogenesis have been made. The damage was cytologically visualized as interchanges between DNA of chromatids or as structural chromosomal aberrations. Whereas non-carcinogenic chemicals had no effects, carcinogenic chemicals at concentrations that are used to induce transformation, caused changes in human and animal chromosome structure by modifying the DNA-nonhistone protein associations which was reflected in induction of G-bands or chromosomal despiralization. The aberrations were visualized in chromosome structures as a deletion of chromosomal material or rearrangements of damaged chromatid materials that usually occurred within one or two cell divisions after carcinogen exposure.

In the course of the above described studies, it was observed that under some situations in cells of some species, such as rat and guinea pig, there are

preferential sites for carcinogen interaction with chromosomal DNA. With several autoradiographic and cytological studies it was found that certain genomic sites are exceptionally vulnerable not only to carcinogens but to damage induced by toxic agents other than carcinogens. In situations such as with Syrian hamsters where no specific involvement of chromosomes or chromosomal segments was found after acute exposures to carcinogens, it was concluded that negative bands of the chromosomes that represent enchromatic regions carrying essential genetic messages are more frequently the target of carcinogen damage than regions containing highly repetitive DNA sequences.

In recent years, new techniques have been developed which result in differentially stained chromatids of a single chromosome and thus enable cytological visualization of response to damage caused by potential carcinogens and mutagens as symmetrical exchanges between sister chromatids (SCE). The SCE's do not result in a change in the overall morphology of the chromosome. Since SCE's involve a breakage and exchange of the DNA duplex in each chromatid which is effected during replication, the extent to which the frequency of SCE's would be useful for detecting carcinogens as well as the factors that influence the formation of SCE's was studied. The latter would obviously be important to understanding the mechanism of both mutagenesis and carcinogenesis. The in vitro response of Chinese hamster and human cells to the induction of SCE's for suspected carcinogens-mutagens as well as a variety of inactive homologs was evaluated. An extensive study, demonstrates that a number of carcinogens including those which require metabolic activation increased the SCE incidence. For chemicals requiring metabolic activation, they were placed on an irradiated layer of cells that normally metabolize a number of chemicals. Non-carcinogens, independent of the protocol used, were negative in terms of induction of SCE's.

Several hypotheses have been formulated to explain the increased frequency of SCE's induced by a variety of agents. The sequence in which a carcinogen and 5-bromodeoxyuridine (BrdUrd) are added to cell cultures affects the number of SCE's induced by a carcinogen. When the cells are first treated with MNNG, there is a statistical increase in SCE's observed at the second division metaphase compared to controls exposed to BrdUrd alone. In contrast, when the BrdUrd incorporation preceded MNNG treatment, the average number of SCE's per metaphase was reduced approximately 75% at the second division cycle. Treating the cells first with BrdUrd may reduce the number of DNA sites available for carcinogen attachment and indicates that the SCE frequency is also dependent upon the number of BrdUrd free strands. However, a relatively low SCE incidence occurred at the second mitosis independent of whether one, two or three BrdUrd substituted strands existed at the time of carcinogen exposure. Because BrdUrd substitution increases condensation or supercoiling in chromatin, probably by modification of chromosomal protein-DNA interaction, it is highly probable that carcinogen-DNA interactions are inhibited by chromatid structure changes.

Whereas Syrian hamster embryo cell transformation proceeds rather rapidly, carcinogenesis utilizing guinea pig cells has been shown to occur in discrete stages that frequently are displayed for extended periods of time. Morphological alteration relative to controls develops shortly after carcinogen

exposure and persists for months. After four or more months, morphologically altered cells proceed to the stage of morphological transformation characterized by random growth and piling up of cells. Neoplastic transformation, the ability of cells to produce tumors when inoculated into host animals, may be demonstrated at the time of morphological transformation or frequently may require additional months of subculture. This stepwise progression of the carcinogenic process occurs following treatment of cells obtained from fetuses in midterm and nearterm gestation and with cells isolated from neonatal guinea pigs. A similar pattern occurs during in vitro carcinogenesis of a non-tumorigenic morphologically oriented fibroblast-like guinea pig continuous cell line treated with carcinogen 14 months after introduction into culture. This indicates that the transformation frequency, latent period, and multiple stages characteristic of carcinogenesis can be independent of cell age or number of generations.

A number of somatic cell alterations including the ability to form colonies in agar, secretion of plasminogen activator, intradermal skin reactivity in nonimmune syngeneic animals and susceptibility to the cytotoxic activity of lymphotoxin, nonimmune macrophages and nonimmune lymphocytes can develop in close proximity or concomitantly with neoplastic transformation. These cell properties provide useful quantitative means for identifying neoplastic transformation of cells in culture and to investigate cellular characteristics essential to the development of the neoplastic state. They also indicate that natural immunity mediated by macrophages, lymphocytes, and the lymphokine, lymphotoxin, recognize and may modify and even inhibit the development of a neoplastic cell.

The neoplastic state is associated with a quantitatively specific susceptibility to lymphotoxin. There is a wide range of lymphotoxin susceptibility among different neoplastically transformed cells that is independent of the origin of the cells or the initiating chemical carcinogen. Neither chromosomal alterations nor detectable tumor specific cell surface neoantigens are necessary for neoplastic transformation; furthermore, these factors are unrelated to lymphotoxin susceptibility. Colony formation in agar and lymphotoxin susceptibility can develop concomitantly with or following the onset of the stage of preneoplastic morphological transformation. They also can develop although rarely independently of one another and many cell generations and months prior to attaining the stage of neoplastic transformation. Natural intradermal skin reactivity, however, has yet to be observed until the stage of neoplastic transformation. This separation of lymphotoxin susceptibility and natural skin reactivity into two different stages of carcinogenesis is of interest as these are two potential points for immunobiologic modulation of in vivo carcinogenesis.

Guinea pig, human and mouse lymphotoxin exhibit different species specificities for neoplastic and nontumorigenic cells. Mouse lymphotoxin and mouse tumor cells appear to have little species specificity. Mouse lymphotoxin is more cytotoxic to tumor than to nontumorigenic cells regardless of the species origin of the cells and mouse tumor cells are susceptible to the cytostatic (colony inhibitory) and cytolethal (radionuclide release) activity of guinea pig, human and mouse lymphotoxins. Guinea pig and human lymphotoxin, however, possess distinctive species specificity that is generally cytostatic rather

than cytolethal. For example, guinea pig lymphotixin inhibits the growth of guinea pig but stimulates the growth of human tumor cells in colony inhibition assays. In contrast, human lymphotoxin stimulates guinea pig and inhibits human tumor cells. Lymphotoxin susceptibility is qualitatively and quantitatively independent of detectable tumor specific neoantigens. Each tumor or tumor cell line, however, exhibits its own characteristic susceptibility to natural immunocytotoxicity as well as its own intradermal skin reactivity in non-immune syngeneic animals. The reasons for these remain to be explained and may provide fundamental new understandings to basic immunology, tumor biology, and to host interactions modulating carcinogenesis.

Lastly, a human fibroblast cell strain (MRC) undergoes neoplastic transformation as a result of treatment with a number of direct-acting chemical carcinogens. Like guinea pig cells, human cells have an extended latent period; but, in addition, the transformation is facilitated when the cells are blocked in G_1/S prior to treatment. The transformed cells form tumors when injected intracranially in nude mice and have an extended life span. Control cultures have a limited life, fail to form colonies in agar, or to produce tumors. Progress has been made in each of the objectives recognized as the Laboratory of Biology's responsibility to the Carcinogenesis Intramural Program of the Division of Cancer Cause and Prevention. Fundamental information has been obtained concerning biological, chemical and physical agents that induce transformation. Furthermore, a number of factors, chemical and biological modifiers, that modulate the transformation process have been identified. Studies have been initiated to determine the mechanism of action of these factors by investigating biochemical changes relevant to the transition to neoplasia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04629-15 LBGY

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Mechanism of Cell Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biology, Carcinogenesis Intramural Program

SECTION
Somatic Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	3.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The conditions which are responsible for modulation of in vitro transformation will be investigated further, with specific emphasis on the use of UV, X-rays, combinations of carcinogens of different classes, the combination of in vivo - in vitro systems, and the effect of repair mechanisms on toxicity and transformation. The relevance of chromosome damage as a result of initial carcinogen insult as well as to which cytogenetic alterations are responsible for or associated with malignancy is being determined using cells from a variety of species including human. Studies on the mechanisms of early events of carcinogen-cell interaction will be continued, as well as investigation of different proximate carcinogens and metabolic inhibitors. Special emphasis will be given to the requirements necessary to obtain neoplastic transformation of human fetal cells. The testing for the tumorigenicity of cells exposed to different agents for different time intervals will continue.

Project Description

Objectives: The primary objective of this project is to establish conditions and methods for in vitro quantitative study of chemical transformation. The immediated targets of study are:

1. Development of rapid assay systems for chemical carcinogens suitable for the screening of compounds and populations.

2. Define the conditions required for the transformation of human fetal cells.

3. Determine ways to increase the susceptibility of primary cell lines or cell strains to chemical transformation.

4. Development of assays to determine whether in vitro transformation is accompanied by the appearance of tumor specific antigens.

5. Determine the role of nononcogenic and oncogenic viruses in chemical transformation.

6. Determination of the ultrastructural changes of cells during the course of in vitro chemical transformation so that they may be contrasted with normal cells and with information pertaining to viral transformation.

7. Determine the role of repair, e.g. rate, excision and post-replication in enhancing neoplastic transformation with radiation and alkylating compounds.

8. The transformation of epithelial-like cells which result in the formation of carcinomas when the cells are transplanted into animals.

9. Analysis of variations in populations of somatic cells and their correlation with genetic changes.

Methods Employed: All procedures performed are with the view of quantitating phenomena in vitro. Such procedures are required to determine whether or not the transformation observed is due to the direct or indirect effect of the carcinogen and in order to study the early events associated with in vitro transformation. Cells used come from freshly isolated cells from animals and humans that as controls have many of the attributes of "normal" cells and from cell lines which are known to exhibit some of the properties associated with nontransformed cells. Discrete cells are grown in complete medium in the presence or absence of irradiated rat or hamster cells. The cells may be derived from whole embryos or may be from specific organs. The cells are exposed to chemical carcinogen transplacentally or prior to or subsequent to seeding the cells in Petri dishes.

Approximately one week subsequent to treatment, the cells are examined under phase or under stained conditions for number of transformed colonies, toxicity, and spectrum of morphology of both the normal and transformed colonies. The frequency of transformation is expressed in a number of different ways. These

take into consideration the observed rate of transformation on a per-cell basis or on a number of colonies obtained.

Major Findings: Several in vitro models have been developed to study the transformation of normal to neoplastic mammalian cells, yet, to date a routine in vitro system to study human cell transformation is not available. A few successful accounts of human cell transformation have been reported, however, the use of cells derived from early passage foreskin or a lip biopsy make the characterization and establishment of a routine assay system virtaully impossible. Current studies have resulted in the transformation of a human embryonic lung cell strain, MRC-5. These cells were grown in minimal essential medium, containing Hepes (25 mM) glutamine (2 mM), $NaHCO_3$ (0.2%) and 10% fetal bovine serum (CM). When the cells reached a confluency of 60-75%, the medium was replaced with Dulbecco's MEM minus arginine and glutamine which arrested a majority of the cells (<90%) at the G_1/S interface. After 24 hr, the cells were returned to the CM which contained insulin and 8-12 hr later were treated with physical or chemical carcinogens. The time of treatment corresponded to the interval when the maximum number of cells were in the S phase of the cell cycle. After carcinogen treatment, the cells were subpassaged for 4-8 population doublings and 10^5 cells were seeded in low calcium Dulbecco's medium containing 0.35% soft agar. After 17 days, the frequency of colony formation in soft agar was determined. Untreated MRC-5 cells did not produce any colony growth in soft agar. The size of the colonies following carcinogen treatment ranged between 50-500 cells. UV irradiation resulted in the highest frequency of colony growth in soft agar (10^{-3}) while aflatoxin-β_1, and propane sultone treatment resulted in colony formation frequencies of 10^{-6}. Similarly, the addition of 4-nitroquinolone, β-propriolactine or N-methyl-N'-nitro-N-nitrosoguanidine to the MRC-5 culture medium produced colony formation frequencies in soft agar of $10^{-4.5}$, 10^{-5} and 10^{-4}, respectively. The frequencies of colony formation after carcinogen treatment reported here are substantially different than those reported by Milo and DiPaolo which suggest that carcinogen-induced anchorage independent growth is dependent on the specific target cells. The colonies growing in soft agar were isolated, disaggregated and grown as mass cultures. Tumorigenicity was assessed by intracranial injection of 5×10^5 cells suspended in minimal essential medium into 2-3 month old nude mice. Five to six weeks later, the nude mice which received the carcinogen-treated MRC-5 cells developed abnormal motor functions such as hyperkinesis, while the animals which received control MRC-5 remained normal. The carcinogen-treated nude mice were killed, brains removed and fixed in formalin for histology. Upon examination of the prepared brain sections from nude mice injected with 5×10^5 β-propriolactone-treated MRC-5 cells a highly invasive, undifferentiated fibrosarcoma was evident. Histological slides prepared from nude mice brains which received control MRC-5 cells and killed at the same time interval revealed normal architecture. Therefore, colony growth in soft agar and the development of undifferentiated fibrosarcomas in brains of nude mice suggest in vitro transformation of human embryonic lung cells (MRC-5) is possible.

To the present time, only limited success in transforming epithelial cells from various species has been reported and none has been the result of a

quantitative transformation. Furthermore, the formation of carcinomas as a result of the transformation of "epithelial cells" has often been equivocable. It is unreasonable to consider that the mechanism for conversion from normal to the transformed state is dependent upon the original cells being fibroblast or epithelial. Nevertheless, the transformation of differentiated epithelial cells should provide specific biological marker associated with differentiates possibly relevant to the process of carcinogenesis and to intervention or prevention of organ specific cancer. Current rat and human mammary cells are being utilized in vitro. As a step towards transforming mammary cells, the metabolism of benzo(a)pyrene (BP) in hormone responsive rat cells has been demonstrated and compared to that which occurs as a result of cocultivation with liver microsomes. Ficoll derived rat mammary epithelial cells (MEC) and liver microsomes were incubated with ^{3}H-BP and the ethyl acetate extractable metabolites were compared using HPLC techniques. Incubation of microsomes with ^{3}H-BP resulted in 31.4% of the total cpm eluting with the 4-5, 7-8, and 9-10 BP dihydrodiols. BP quinones and phenols accounted for 15.7 and 49.5% of the cpm. After 3-methylcholanthrene (MCA) or 7,12-dimethylbenz(a)anthracene (DMBA) pretreatment, the ratios of BP metabolites remained unchanged, but BP metabolism increased 16.9 and 4.5-fold, respectively. Whereas with untreated MEC 67.4% of the metabolites produced eluted with BP dihydrodiols, 30% with BP phenols and none with BP quinones. Moreover, of the dihydrodiols produced, 42.4 and 25.0% cochromatographed with BP-9,10- and BP-7, 8-diols, whereas BP-4,5-diol was not detected. After MCA pretreatment of MEC, 24% of the cpm was equally divided among the three dihydrodiols with 6.4 and 15.6% of the extractable cpm coeluting with BP quinones and phenols. A large amount of highly polar BP metabolites, amounting to 40.2% of the total cpm, was also produced. With DMBA pretreatment, 43.0% of the cpm coeluted with the BP dihydrodiols, whereas quinones, phenols and highly polar derivatives cochromatographed with 8.3, 29.5, and 17% of the extractable BP metabolites. When 4 day-old primary MEC cultures were treated with DMBA (1 μM) for 24 hr and grown for 8 days in hormone supplemented medium, a colony frequency in soft agar of 10^{-5} was obtained. Thus, rat MEC produce a larger variety of highly polar BP metabolites compared to liver microsomes and their growth is anchorage independent after DMBA treatment in vitro.

Asbestos, a special group of naturally occurring silicate mineral fibers, is found in thousands of commercial products. Numerous epidemiologic and experimental animal studies, have established the cancer-producing potential of asbestos. Tissue culture studies have been limited for the most part to determining relative toxicity of the fiber types and have concentrated on mechanisms involving microphages stimulated by asbestos to secrete plasminogen activator. Since transformation of fibroblasts is relevant to carcinogenicity, the effect of asbestos fibers on cloning efficiency and transformation of Syrian hamster cells alone and in combination with benzo(a)pyrene (BP) per ml medium was determined. The asbestos fibers used are classified as crocidolite, crysotile, emosite, and anthophyllite. The largest fibers, erysolite, were the most toxic. Preliminary experiments demonstrate that whereas BP and asbestos both reduced the cloning efficiency relative to untreated cells, the transformation obtained by BP was much greater than that obtained by asbestos fibers alone. In fact, asbestos by itself produced only rare transformation

of the order of 0.1-0.5 transformations per dish as opposed to BP that induced over one transformation per dish. Interestingly, the addition of BP and asbestos to cells plated for colony formation did not increase the toxicity as indicated by the cloning efficiency; however, the average transformation per dish did increase synergistically and, depending upon the experiment, ranged from 4 to 6 per dish on an absolute basis with crysotile and BP inducing the most transformation. Therefore, one can conclude that asbestos can actually enhance the transformation generally associated with BP. Substitution of ultraviolet irradiation for BP was ineffective in the sense that no enhancement of transformation was observed.

Cells transformed to the neoplastic state by chemical carcinogens have been shown to be sensitive to the cytotoxic and cytocidal activity of soluble factors referred to as lymphokines, lymphoid cell products. Lymphokines do not require individually specific antigens on tumor cells for lymphokine induction or action. For example, the susceptibility of guinea pig cells to the colony-inhibitory activity of lymphotoxin occurs when the cells have neoplastic properties. A lymphokine, lymphotoxin obtained from non-immunized hamsters, is being evaluated for anticarcinogenic activity using the quantitative in vitro hamster colony transformation model. Lymphotoxin was produced by mitogen stimulation of Syrian hamster mineral oil induced peritoneal leukocytes by incubation of the cells with 1000 µg of phytohemagglutinin/10^7 cells in 10 ml medium/100 mm dish for 24 hr. Secondary subcultures from fetal cells were seeded (300 cells/60 mm dish) for colony formation with irradiated cells (feeder layer) and treated with a carcinogen 24 hr later. Aliquots of the cell-free lymphokine culture medium containing lymphotoxin were added simultaneously with carcinogen or at 1, 2, or 4 days post carcinogen. Transformed colonies were scored 6 days after carcinogen exposure. Lymphotoxin did not induce transformation or affect cell survival as indicated by cloning efficiency in non-carcinogen treated cultures. Transformation decreased in proportion to lymphotoxin concentration in cells treated with benzo(a)pyrene (BP), N-methyl-N'-nitro-N-nitrosoguanidine, N-acetoxyacetylaminofluorene or 254 nm ultraviolet with doses that induce 2-5 transformed colonies per dish. The decrease in transformation frequency from 0 to 100% over a 500-fold range of lymphotoxin was independent of time of addition of lymphokine during the first 4 days of carcinogen exposure. The number of transformed colonies remained constant in carcinogen-lymphotoxin treated dishes refed with lymphotoxin-free medium: e.g. transformation frequency was reduced by 50% in cells refed with medium 3 days after addition of 20 units lymphotoxin and 2.5 µg BP/ml medium and in cells incubated for 6 days with lymphotoxin and BP. Thus, lymphotoxin, a naturally occurring biological molecule, inhibits rather than suppresses the transformation process.

It is commonly accepted that the phenomenon of carcinogenesis precedes through a series of stages which can be studied by observing carcinogenic effects. A special category of cocarcinogenic response is exemplified by croton oil or its active principle, 12-O-tetradecanoyl-phorbol 13-acetate (TPA), which by itself is considered to be noncarcinogenic and effective only when applied after a subeffective dose of carcinogen. The effect of the tumor promoter TPA on chromosomal alterations induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in

Chinese hamster line (V79) and in secondary Syrian hamster embryo fibroblast (HEC) cultures and on transformation using a quantitative Syrian hamster model in which 300 target cells are cocultivated with irradiated cells/60 mm dish and treated commencing 24 hr post seeding was determined. Nontoxic concentrations of TPA (1-1000 ng/ml medium) neither altered the frequency of sister chromatid exchanges (SCE) in control cells nor in those pretreated with MNNG (0.25 μg/ml medium) independent of whether logarithmically or stationary cultures were treated and whether TPA was added immediately after or 24 hr post carcinogen. MNNG induces chromatid gaps and breaks with larger chromosomes mostly involved. The increase in the frequency of chromosomal aberrations induced by MNNG and TPA was not statistically significant. Chromatid exchanges (triradials/quadriadials) and total number of aberrations increased ∿50% whereas the percent of cells with aberrations was comparable. While no transformation occurs with 1000 ng TPA/ml medium, TPA effect on MNNG induced transformation is time dependent. TPA 24 or 48 hr prior to MNNG did not alter the transformation obtained by MNNG alone but TPA 2 hr after MNNG reduced transformation frequency by 50%. However, when TPA, 10-100 ng/ml medium was added 24-72 hr post MNNG (0.1 or 0.25 μg/ml medium), the absolute transformation increased up to 5-fold. These experiments suggest that threshold concentration of carcinogen initiate many more cells that are completely transformed. This frequency would argue against a point mutation as the initial cause for transformation and suggests that originally the transformation frequency is underestimated. The lack of effect of TPA on MNNG induced SCE indicates that increased in vitro transformation does not involve a SCE type of mitotic recombination. Increase in exchange type aberrations may reflect TPA stimulation of recombination enzymes presumably activated by MNNG and correlates with reduced transformation found when TPA is added 2 hr post MNNG.

Recently, protease inhibitors have been the subject of studies concerned with inhibition of experimental tumorigenesis and cell transformation by 3-methylcholanthrene or X-irradiation. The in vivo experiment was done using a two-stage carcinogenesis model in which a subcarcinogenic dose of carcinogen is considered the initiator and repeated applications of 12-O-tetradecanoylphorbol-13-acetate (TPA), a noncarcinogenic promotor, resulted in the induction of a trypsin-like protease in mouse skin. When protease inhibitors followed the application of TPA in the two-stage carcinogenesis model, inhibition of tumorigenesis resulted. Syrian golden hamster cell strains areparticularly appropriate for investigating the role of antipain in inhibiting transformation in vitro and its relevance to chromosomal changes. These non-malignant cells have a stable chromosomal karyotype and the analysis of G-banded chromosomes after N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment showed that chromatid types of aberrations considting of gaps, breaks and exchanges occur. Although no specificity in terms of involvement of chromosome or chromosome segments are noted, larger chromosomes appeared more often to be the target of MNNG damage. In terms of transformation results, a dose-response relationship is reproducibly demonstrable with MNNG. The morphological transformation induced in Syrian hamster embryo cells by MNNG (0.25 μg/ml medium) is inhibited by post treatment with antipain (6-600 μg/ml), a protease inhibitor. DNA replication relative to untreated controls is not affected by MNNG, antipain,

or the combination of the two; no synergistic lethality of antipain and MNNG occurred as reflected in the cloning efficiency. Pretreatment with antipain 24 hr before MNNG did not alter the transformation frequency obtained with MNNG. Whereas MNNG significantly increased the frequency of sister chromatid exchanges (SCE), antipain was ineffective in influencing carcinogen induced SCE. An increase in chromosome aberrations (per metaphase) occurred at 10, 26, and 40 hr when cells were treated with 0.25 μg MNNG/ml medium followed by antipain 10 min later, the procedure used in the transformation studies; antipain also increased the average number of aberrations at the second mitosis (34 hr) when the concentration of MNNG was doubled. An increase in chromatid exchanges occurred 26 hr post-treatment with the combination of MNNG and antipain used for transformation. No difference in MNNG induced aberrations was observed when antipain preceded MNNG by 24 hr. Since the mode of action of antipain is unknown, the question remains whether its effect on transformation results from inhibition of derepression of particular genes necessary for transformation or from elimination of some cells, because of lethal chromosome damage, that would ordinarily undergo transformation.

While the molecular basis for carcinogenesis is still largely unknown, DNA repair appears to play an important role. This is inferred from the fact that patients, with rare genetic disease such as xeroderma pigmentosum and ataxia telangectasia, are both cancer prone and have a genetic DNA repair defect. How DNA repair is involved in carcinogenesis is an enigma. To understand the relationship between DNA repair and carcinogenesis, coordinated studies of DNA repair and in vitro transformation of Syrian hamster embryo cells are being done. Secondary subcultures were seeded (300 cells/60 mm dish) with X-irradiated feeder cells for colony formation 1 day before UV and caffeine treatment. The frequency of UV-induced transformation was enhanced 2 to 5-fold when the cells were incubated for 48 hr with caffeine (0.25 mM) after UV (254 nm) irradiation ($3J/m^2$). Maximum enhancement occurred when caffeine was added to the cells 4 hr post UV. After cells are UV irradiated, two modes of DNA replication are observed. The size of pulse labeled nascent strands in cultured cells was measured by alkaline sucrose sedimentation. During the early mode that operates for the first 3-4 hr post UV, nascent DNA strands are smaller than in non-irradiated cells. In the late mode, the nascent strands recover to normal size. During the early mode, the time required for nascent strands to elongate to parental size was about 3 times longer with caffeine than without. Furthermore, when caffeine (1 mM) was added immediately after UV irradiation ($10J/m^2$), the conversion of the early mode of replication to the late mode was inhibited. The conversion from early mode to late mode replication occurs at about 4 hr post UV, when addition of caffeine increases transformation the most. When caffeine was added 4 hr after UV irradiation, the size of nascent strands replicated in the next 3 hr was increasingly smaller. Thus, caffeine caused the late mode of replication to revert to the early mode. The observed caffeine induced changes in the post UV DNA replication can account for the potentiation of UV-induced lethality and may be involved in the enhancement of transformation.

Syrian hamster embryo cells can be transformed in vitro to the neoplastic state by exposure to the ultraviolet light (UV) emitted by a germicidal lamp. A UV

action spectrum was performed to determine the target involved in this photobiological interaction. DNA has been shown to be the target for both UV-light induced cell killing and mutagenicity of the bacterium, Eschericia coli, because the action spectrum matched the DNA adsorption spectrum. The action spectrum for cytotoxicity, in vitro transformation, and production of pyrimidine dimers (the major Uv photoproduct) was measured to determine the target for the initiation of carcinogenesis. Monochromatic light was produced by a double grating monochrometer containing a Xenon Mercury lamp. Cytotoxicity and transformation were determined by plating 300 Syrian hamster embryo cells per 60 mm dish, irradiating the cells, and allowing 7 days total incubation. The dishes were stained and the number of morphologically transformed colonies as well as the total number of colonies were scored. Pyrimidine dimer production was measured by determining the frequency of sites in DNA extracted from irradiated cells sensitive to a pyrimidine dimer specific endonuclease. The effect of at least three different fluences of UV at each of the following wavelengths (nm) were measured: 240; 254; 265; 270; 280; 290; 297; 302, and 313. For each wavelength, the fluence that had the equivalent biological effect as a fluence of $6J/m^2$ at 254 nm was determined. The result of dividing $6J/m^2$ by the above fluence gives the relative effectiveness for the given wavelength. The relative effectiveness for cytotoxicity, transformation, and pyrimidine dimer production were found to be essentially the same at each of the wavelengths tested. Furthermore, the most effective wavelengths were 265 and 270 nm. This is the same range as has been determined by others as the maximum for the production of chromosome aberrations and mutations in mammalian cells. The maximum adsorption of DNA and protein are 260 and 280 nm, respectively. Since the wavelength for maximum pyrimidine production and biological effectiveness is in the range of 265 to 270, both DNA and protein could be chromophores for these photobiological interactions. However, the target for pyrimidine dimer production can only be DNA. Since the action spectra for cytotoxicity, mutation, aberration production, and most importantly transformation, are the same as that for pyrimidine dimer production, this suggests that DNA is also the target for these processes.

Syrian hamster cells are important for cytogenetic studies in carcinogenesis, mutagenesis and teratogenesis. The chromosomal characteristics of this species have been determined to facilitate the identification of abnormal chromosomes from neoplastic cells. C- and Q-banding karyotype was established, the location of heterochromatin and the regions associated with fibrosomal cistrons coding for 18 and 28 S RNA were determined. Syrian hamster chromosomes contain an unusually large amount of constitutive heterochromatin of two types, centromeric and noncentromeric. To determine the changes in heterochromatin and euchromatin in evolution, the chromosome of the species comprising the genus Mesocricetus, M. auratus (2n=44), two "cryptic" species of M. brandti (2n=44 and 2n=42) and M. newtoni (2n=38) were investigated using several cytological methods for chromosome banding, heterochromatin identification and for visualization of the regions associated with the nucleolar organizer function (NOR). Most of the autosomes and sex chromosomes have either a complete or partial G-band correspondence and thus providing definitive evidence for the taxonomic relationship of these species. The karyotypic differences identified occurred primarily as a result of deletion of sex

chromosome heterochromatin and autosomal translocations that frequently involved chromosomes with heterochromatic short arms or chromosomes bearing NOR. Only one Robertsonian centric fusion occurred. However, changes in heterochromatin occurred during the evolution of these species. Cytological evidence for the evolution of these species was obtained. M. brandti and newtoni each have a metacentric chromosome which shares a common long arm matching a submetacentric M. auratus autosome but a short arm corresponding to two different M. auratus chromosomes. These translocations are crucial for explaining the direction of chromosomal evolution of these species and indicate that M. auratus was the common ancestral species and that M. brandti and newtoni subsequently evolved independently.

At early passages, after recognition of Syrian hamster cell foci transformed by SA7, a normal diploid or near diploid constitution with minor structural or numerical deviations occurs. Recently, a number of virus-transformed foci selected from dishes pretreated with chemical carcinogens and established as cell lines in culture were shown to contain equivalent amounts of SA7 viral genome, approximately 1-2 per cell. A number of these virally-transformed cell lines have been re-examined with high resolution radioautography, in conjunction with in situ molecular hybridization and sister chromatid exchange (SCE) techniques, to determine the extent to which the viral genome is integrated on specific chromosomes and the consequences of such integration on SCE frequency. For in situ hybridization, unstained air-dry preparations and destained slides that had been processed for G-bands were used. The ^{3}H RNA-DNA hybridization technique of Pardue and Gall for conventional cytological preparations was used with minor modifications. Labeled nuclei and metaphases were scored on randomly selected areas of the slides. For SCE analysis, bromodeoxyuridine (10 µg/ml) was added to the culture medium 26 hr before chromosome fixation and cells were maintained in complete dardness until the chromosome fixation. Although cell lines had detectable SA7 "T" antigen and were tumorigenic, the incidence of labeled nuclei and metaphases ranged from 0 to 60%. Transformed lines examined soon after transformation had more label than others examined relatively late. No consistent accumulation of label occurred in a specific chromosome region. In situ hybridization was less efficient than molecular hybridization, but not even cells with high SA7 DNA content showed chromosome specificity for label. Therefore, no correlation exists between ^{3}H RNA label and specific chromosome or chromosomes of neoplastic cells. Some cell lines had a higher incidence of SCE, possibly reflecting the integration of the SA7 genome in some transformed lines.

A positive relationship has been shown between induction of sister chromatid exchange (SCE) and mutation with some chemical carcinogens using malignant cells. We examined the relation between SCE and transformation by quantifying in parallel SCE and transformation induction in Syrian hamster cells with several carcinogens that differ in their mode of interaction with DNA. Although the relative efficiency of SCE and transformation induction varied with the carcinogen used, a positive dose dependent relation in SCE and transformation was observed with N-methyl-N'-nitro-N-nitrosoguanidine, benz(a)pyrene, N-acetoxy-2-acetylaminofluorene and cis Platinum(II)diamine dichloride. Methylmethanesulfonate caused a significant increase in SCE but induced only a few

transformations. The regression line had a slope significantly different from 0 for all chemicals tested. On the other hand, X-ray irradiation (200-500R) failed to increase SCE or to transform the cells. The response for SCE and transformation induction to carcinogen treatment at the same concentrations exhibit a similarity in the sensitivity for the induction of SCE and transformation.

When cells are pulsed with a carcinogen, chromosome and subchromatid alterations are induced. The latter involve reciporcal interchange of DNA between chromatids and can be cytologically visualized as sister chromatid exchanges (SCE). The inducible SCE frequency in cell strains and cell lines of human (normal leukocyte cultures vs lymphoblastoid cell line HH4), Syrian golden hamster (fetal secondary cultures vs BHK cell line), Chinese hamster (fetal secondary cultures vs V79-4 cell line), was compared using the 5-bromodeoxyuridine technique. In all species examined, the permanent cell lines responded with a higher frequency of N-methyl-N'-nitro-N-nitrosoguanidine (0.25 µg/ml medium) induced SCE compared to the cells derived from fresh normal tissues. The increased sensitivity in terms of SCE induction may reflect either a more sensitive or a different DNA repair capacity of normal cells. On the other hand, a failure of some type of DNA repair which results in a reduced removal of carcinogen induced DNA damage may be responsible for a higher SCE frequency such as observed in permanent cell lines.

Significance to Biomedical Research and the Program of the Institute: The prevention of cancer in humans depends to a large extent on understanding the process that is responsible for the development of transformation and on removing potentially harmful environmental agents. The determination of factors responsible for transformation serve as basis for the study of mechanism(s) involved in transformation. In this way, it will be possible to intervene with or prevent the development of cancer. The cellular approach with human and animal cells will make it possible to study how to block, reverse, or eliminate transformed cells. In the process, biologically valid techniques for identifying potential carcinogens relevant to humans will also be developed.

Proposed Course of the Project: The overall approach of the Laboratory of Biology to problems in carcinogenesis will continue to be to investigate the modulation of transformation of mammalian cells, vital aspects in the etiology and prevention of cancer. Whereas cell biology was emphasized in the past, molecular biological approaches are increasingly important in the elucidation of the objectives of the laboratory. Our specific objectives are (1) to define the role of chemical, physical and biological agents pertinent to the process of carcinogenesis, (2) to characterize the cellular alterations associated with carcinogenesis, (3) to evaluate the relationships between DNA (chromosome) and carcinogenesis, and (4) to probe the somatic mutation aspects of experimental carcinogenesis.

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October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Immunobiology of Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Natural immunity can inhibit the development of a tumor cell during carcinogenesis as evidenced by cytostatic and cytocidal assays during chemical carcinogen induction of neoplastic transformation in cultured mammalian cells. Natural cytotoxic activity of normal unstimulated nonimmune macrophages and lymphocyte populations for tumor cells is quantifiable by lymphoid effector cell mediated inhibition of tumor cell colony formation and release of radionuclides. Lymphotoxin, one of the lymphokines, soluble mediators of immunoregulatory function, also exhibits cytotoxicity for tumor cells not observed with nontumorigenic cells. In in vitro models of carcinogenesis such as neoplastic transformation of guinea pig cells where distinct discrete stages in carcinogenesis can be readily identified and studied, susceptibility to natural immunocytoxicity develops concomitantly or in close association with neoplastic transformation. Neoplastic cells but not their nontumorigenic counterparts also exhibit delayed hypersensitivity skin reactions in normal nonimmune syngeneic animals. This natural skin reactivity like natural immunocytotoxicity is independent of cell surface tumor specific transplantation rejection antigens yet is individually distinctive for each tumor cell line.

Project Description

Objectives: The primary objective of this project is to elucidate at the target cell and host levels the relationships between cell surface alterations accompanying the development of neoplasia in the phenomenon of carcinogenesis. Specific objectives include: (1) the identification of somatic cell alterations during carcinogenesis using *in vitro* model systems to allow study of membrane and other phenotypic changes at specific steps or stages in carcinogenesis, and (2) investigation of host interactions with specific cell surface alterations during carcinogenesis *in vitro* and *in vivo*. Particular emphasis is placed on natural and induced cellular and humoral immunobiological interactions due to the frequent occurrence of neoantigens, reexpression of fetal antigens, and alterations in alloantigens on tumor cells. Investigations have concentrated in the past on somatic cell alterations developing during discrete stages in carcinogenesis identified during the development of a guinea pig *in vitro* model of carcinogenesis in the Laboratory of Biology. Present and future investigations have been expanded to include a variety of *in vitro* and *in vivo* systems to broaden the observation base of the project.

Methods Employed: Guinea pig, human, mouse, rat, and Syrian hamster cells are utilized in this study. The quantitative *in vitro* chemical carcinogenesis system developed within the Laboratory of Biology employing Syrian hamster embryo cells (Project ZO1-CP-04629, The Mechanism of Cell Transformation) forms the basic methodology for obtaining normal, preneoplastic and neoplastic cells for study. Freshly isolated cells are obtained from whole or specific organs of embryos, fetuses or adult animals, exposed *in utero* or *in vitro* to chemical or physical carcinogens, cultured, and studied for somatic cell changes such as altered morphology, morphological transformation, growth in agar, tumorigenicity and interaction with components of the immune system. Immunobiological techniques including direct and indirect immunofluorescence, complement fixation, colony inhibition, radionuclide uptake and release assays, delayed hypersensitivity skin reactions, and tumor transplantation rejection are employed in analyzing cell membrane changes and in assessing host interactions to the changes.

Major Findings:

1. Carcinogenesis utilizing guinea pig cells proceeds in discrete stages that frequently are displayed for extended periods of time. Morphological alterations relative to controls develop shortly after carcinogen exposure and persist for months. After four or more months, morphologically altered cells proceed to the stage of morphological transformation characterized by random growth and piling up of cells. Neoplastic transformation, the ability of cells to produce tumors when inoculated into host animals, may be demonstrated at the time of morphological transformation or frequently may require additional months of subculture. This stepwise progression of the carcinogenic process occurs following treatment of cells obtained from fetuses in midterm and near-term gestation and with cells isolated from neonatal guinea pigs. A similar pattern occurs during *in vitro* carcinogenesis of a non-tumorigenic morphologically oriented fibroblast-like guinea pig continuous cell line treated with

carcinogen 14 months after introduction into culture. This indicates that the transformation frequency, latent period, and multiple stages characteristic of carcinogenesis can be independent of cell age or number of generations.

2. A number of somatic cell alterations including the ability to form colonies in agar, secretion of plasminogen activator, intradermal skin reactivity in nonmimmune syngeneic animals and susceptibility to the cytotoxic activity of lymphotoxin, nonimmune macrophages and nonimmune lymphocytes develop in close proximity or concomitantly with neoplastic transformation. These cell properties provide useful quantitative means to identify neoplastic transformation of cells in culture and to investigate cellular characteristics essential to the development of the neoplastic state. They also indicate that natural immunity mediated by macrophages, lymphocytes, and the lymphokine, lymphotoxin, recognizes and may modify and even inhibit the development of a neoplastic cell.

3. The neoplastic state is associated with a quantitatively specific susceptibility to lymphotoxin. There is a wide range of lymphotoxin susceptibility among different neoplastically transformed cells that is independent of the origin of the cells or the initiating chemical carcinogen. Neither chromosomal alterations nor detectable tumor specific cell surface neoantigens are necessary for neoplastic transformation; furthermore, these factors are unrelated to lymphotoxin susceptibility. Colony formation in agar and lymphotoxin susceptibility can develop concomitantly with or following the onset of the stage of preneoplastic morphological transformation. They also can develop although rarely independently of one another and many cell generations and months prior to attaining the stage of neoplastic transformation. Natural intradermal skin reactivity, however, has yet to be observed until the stage of neoplastic transformation. This separation of lymphotoxin susceptibility and natural skin reactivity into two different stages of carcinogenesis is of interest as these are two potential points for immunobiologic modulation of in vivo carcinogenesis.

4. Guinea pig, human and mouse lymphotoxin exhibit different species specificities for neoplastic and nontumorigenic cells. Mouse lymphotoxin and mouse tumor cells appear to have little species specificity. Mouse lymphotoxin is more cytotoxic to tumor than to non-tumorigenic cells regardless of the species origin of the cells and mouse tumor cells are susceptible to the cytostatic (colony inhibitory) and cytolethal (radionuclide release) activity of guinea pig, human and mouse lymphotoxins. Guinea pig and human lymphotoxin, however, possess distinctive species specificity that is generally cytostatic rather than cytolethal. For example, guinea pig lymphotoxin inhibits the growth of guinea pig but stimulates the growth of human tumor cells in colony inhibition assays. In contrast, human lymphotoxin stimulates guinea pig and inhibits human tumor cells. Lymphotoxin susceptibility is qualitatively and quantitatively independent of detectable tumor specific neoantigens. Each tumor or tumor cell line, however, exhibits its own characteristic susceptibility to natural immunocytotoxicity as well as its own intradermal skin reactivity in nonimmune syngeneic animals. The reasons for these remain to be explained and may provide fundamental new understandings to basic immunology, tumor biology, and to host interactions modulating carcinogenesis.

Significance to Biomedical Research and the Program of the Institute: This project provides a means to study and understand how the individual through the mechanisms of natural immunity intervenes to suppress, inhibit or even enhance the growth of an incipient tumor cell during carcinogenesis. Natural cytotoxicity of macrophages, lymphocytes and lymphokines alone or in combination can now be studied at various stages of carcinogenesis to provide new insights into the immunobiology of cancer. As the host mechanisms and the target cell structures with which the immune effectors interact are delineated, it will be possible to investigate how the natural and induced immunity of the individual host may be augmented to suppress and even prevent the final aspects of carcinogenesis, the transition from the preneoplastic to the neoplastic state.

Proposed Course of the Project: Investigations will continue to define the mechanisms whereby neoplastically transformed rodent and human cells are preferentially susceptible to the cytotoxic activity of naturally immune host derived cellular and humoral effectors. A major thrust will be a multidisciplinary investigative analysis of cell membranes relevant to differentiating tumor cells from normal cells. The approaches will include membrane structure changes and macromolecular differences in cellular behavior. This program will in part seek to explain how lymphotoxin preferentially binds to and inhibits tumor cells and why cell to cell contact is necessary in most natural cellular immunocytotoxicity. With the arrival of additional professional staff in the forthcoming and future years, deeper molecular investigation of altered histocompatibility antigens, other surface glycoproteins and glycolipids, as well as pertubations in fibronectin, fibrinolysin and additional cellular components contributing to the intercellular matrix will be possible. New technology in cell surface topography and cell separation using computerized cytometers and cell sorting cytofluorographs will enable resolution of the relationships between the cytostatic and cytocidal activities of macrophages, lymphocytes, and lymphokines to tumor cells. Definition of these relationships will clarify the species specificity of the effector mechanisms and our understanding of the role of *in vivo* natural immunity in the phenomenon of carcinogenesis.

Publications

Rundell, J. O., Evans, C. H.: Biological characterization of guinea pig lymphotoxin production. *Immunopharmacology* 2: 19-27, 1979.

Evans, C. H., DiPaolo, J. A.: *In vitro* mammalian cell transformation for identification of carcinogens, cocarcinogens and anticarcinogens. In Stich, H. and San, R. H. C. (Eds): *Short-Term Tests for Chemical Carcinogens*. New York, Springer-Verlag (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05145-01 LBGY

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cell Surface in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Biology, Carcinogenesis Intramural Program, DCCP

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2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cell surface factors are involved in formation of progressively growing tumors as evidenced by altered expression of normal and coincident appearance of new cell surface properties with tumorigenicity. Plasminogen activator, a protease, is studied in relation to the cell's tumorigenicity and to other frequently altered properties. Guinea pig carcinogenesis in vitro is used to establish the relationship of individual and combined factors to the preneoplastic sequential stages which may precede neoplastic transformation, the acquisition of tumorigenicity, by two years. In this way, the stages and factors associated with carcinogenesis are studied separate from those properties found only in tumorigenic cells. All tumorigenic cells produce plasminogen activator with the amount produced correlating with the (1) tumorigenic threshold inoculum, (2) capacity for anchorage independent growth, (3) ability to induce a tuberculin-like reaction in non-immune guinea pigs, (4) susceptibility to guinea pig lymphotoxin, and (5) reduced expression of cell surface glycolipid (Forssman antigen). Factors whose expression do not correlate with either plasminogen activator production or tumorigenicity include fibronectin production and individually distinct tumor associated cell surface neoantigen expression.

Project Description

Objectives: The objectives of this project are (1) to identify at the molecular level changes occurring as cells progress through the stages of carcinogenesis, (2) to study the inter-relationships among these changes for the purpose of identifying conditions of phenotypic expression necessary for tumor growth, and (3) to relate the essential phenotypic changes to specific alterations in the regulatory mechanisms induced by chemical carcinogens. Emphasis is placed at the initial stage of research on those changes which occur on the cell surface or in the immediate extracellular area and which appear to be related to a variety of transformation associated properties of neoplastic cells. Specifically the research focuses on morphological changes, alterations in membrane composition, extracellular lytic activity, loss of anchorage dependence, susceptibility to natural host defenses and development of means to circumvent host immunological responses.

In the guinea pig in vitro carcinogenesis system several distinct and extended stages in the progression to neoplastic transformation can be identified following treatment with chemical carcinogenic agents. Cells from each stage can be isolated and maintained for study. During the steps of this progression changes occur in the expression of many cell properties and their combined effect confers the capacity to form a progressively growing tumor. Since no single molecular alteration has been identified which by itself is sufficient for tumorigenicity, it is likely that the importance of each change lies in its relationship to the other changes which have occurred. In this study, each change in cell properties is viewed with regard to its appearance during the stages of pre-neoplastic progression and its relationship to other changes which have occurred.

Methods Employed: Guinea pig cells treated with chemical carcinogens have been prepared and cells isolated at various stages along the pathway toward neoplastic transformation. These cultures, many of which have been preserved in liquid nitrogen, are a major source of material for this project. Cells are assessed for (1) the occurrence of morphological changes, (2) capacity to grow as colonies in 0.3% agar, (3) tumorigenicity in syngeneic guinea pigs or other appropriate animals, (4) ability to elicit a sustained intradermal tuberculin-like reaction in syngeneic immunologically compatible non-immune animals, (5) sensitivity to the colony inhibitory effects of lymphotoxin, (6) Forssman antigen and other glycolipid expression, (7) production of fibrinolytic activity, (8) fibronectin, and (9) tumor-associated cell surface antigens. Fibrinolytic activity as a measure of plasminogen activator production is determined by digestion of ^{125}I-labeled fibrinogen. Fibronectin and other cell surface antigen expression are assessed with xenoantibody in a ^{125}I *Staph.* protein A binding procedure or by indirect immunofluorescence. Lymphotoxin susceptibility is determined by inhibition of tumor cell in vitro colony formation.

Major Findings: The fibrinolytic activity of guinea pig cell lines and their sensitivity to guinea pig lymphotoxin varies directly with the number of cells required to produce a progressively growing tumor in syngeneic guinea pigs.

These properties appear to be closely related to the tumorigenic capacity of the transformed cells and may be among the important determinants of tumor growth. The tumorigenic cells, but not the non-transformed or morphologically transformed non-tumorigenic cells, produce a sustained intradermal tuberculin-like response in non-immune syngeneic animals. This response too, appears to directly correlate with the tumorigenic capacity and may represent, like the sensitivity to lymphotoxin, an aspect of the host natural defenses against tumor cells. Also, all the tumorigenic cells form colonies in 0.3% agar whereas the non-tumorigenic cells did not express this property, and the non-tumorigenic cells exhibit a markedly greater expression of Forssman cell surface glycolipid than do the tumorigenic cells. Fibronectin is produced in greater amounts by non-tumorigenic cells but this difference alone is neither large enough nor sufficiently consistent for tumorigenic cells to be distinguished from non-tumorigenic cells. Likewise, unique, cell line distinctive, tumor associated neoantigen expression is not a distinguishing feature since not all the tumorigenic cells express individually distinct surface antigens detectable by tumor transplantation protection tests, by delayed-type hypersensitivity skin tests, and by antisera from syngeneic and xenogeneic animals immunized with the tumorigenic cells.

Significance to Biomedical Research and to the Program of the Institute:
Many cell surface molecules or associated activities are altered on tumor cells. When these alterations occur as a consequence of chemical carcinogen treatment, they do not reflect the introduction of new genetic material but rather are due to disturbances in the regulation of normal gene products or are due to a direct action of the chemical agent on structural or regulatory genes. Altered cell surface expression is being studied with immunochemical and physical biochemical techniques capable of identifying and isolating specific molecular components that can be functionally, as well as physicochemically, identified and their expression related to changes in specific regulatory components. This work is being done using the guinea pig carcinogenesis model with its extended stages of carcinogenesis and the Syrian hamster model of carcinogenesis where the same progression occurs over a more condensed period. Investigations underway concern the role of carcinogen treatment in the immediate and heritable expression of cell surface neoantigens and the altered expression of normal cell surface components, the range of individuality found, and any repetitive occurrences which may indicate the limits of the variations generated and the role of specific interacting factors. Later work will approach the molecular nature of selected components and possible effects of their expression on the immuno-regulatory mechanisms of the tumor-bearing host.

These studies will determine the importance of specific tumor cell surface and intracellular factors in enabling progressive in vivo growth of tumor cells. Identification of these factors and the mechanisms of their functions and interrelations will provide insight into the necessary cellular changes which occur during carcinogenesis. Through this insight, points of intervention may be identified at which the carcinogenesis process may be halted or at which tumor growth may be reversed.

Proposed Course of the Project: Investigations will continue which focus on mechanisms through which cells acquire their ability to form progressively growing tumors. The current base of data will be expanded through the study of additional properties whose expression may be disturbed as a consequence of alterations in the genetic regulatory mechanisms, and the data will be extended to other cell lines and species. Those properties found to be most closely related to tumorigenicity will be further investigated.

SUMMARY REPORT

LABORATORY OF CARCINOGEN METABOLISM

October 1, 1979 through September 30, 1980

"Plans, develops and conducts a research program including (1) identification, chemical analysis, mode of formation, and metabolism of various classes of chemical carcinogens; (2) studies on the toxicology and metabolic pathways of carcinogens in selected animal systems and in relation to man; (3) correlation of chemical and toxicological data for the selection and screening of chemical carcinogens; and (4) development of biological and biochemical methods for the identification of reactive carcinogenic metabolites."

The Laboratory of Carcinogen Metabolism (LCM) is involved in various intramural research projects and to a minimal extent in the scientific direction of some collaborative projects, most of which will conclude shortly. Staff of the laboratory are still involved in the selection of compounds for nomination to the National Toxicology Program and also act on the chemical nomination committee of the National Toxicology Program. Various staff members serve as project monitors for the program at FCRC, act as consultants for various national and international activities in the area of chemical carcinogenesis and environmental carcinogens, furnish advice to other government agencies, to the industrial or academic world, and serve on various panels, boards or commissions to deal with problems of chemical carcinogenesis.

Research activities in the Office of the Chief are directed largely towards studies on metabolism or activation of various classes of chemical carcinogens, mostly aromatic amines and dialkylnitrosamines. Some small scale bioassays using the strain A mouse lung adenoma test are monitored. Physical-chemical differences between normal and transformed cells are also investigated.

With respect to aromatic amines, studies on the metabolism of 2,4-diaminoanisole in hamsters and comparing these data with that in rats have shown that 1-methoxy-2-amino-4-acetamido-5-hydroxybenzene presumably is excreted as a glucuronide in the urine of both rats and hamsters. However, during the isolation processes it is highly probable that oxidation occurs to afford a large quantity of 2-acetamido-5-methoxy-1,4-benzoquinone. Further studies into the mechanism of this reaction are being investigated.

It was found that various inducers of P_1-450 such as 3-methylcholanthrene, TCDD and β-naphthoflavone increased the levels of certain metabolites of 2,4-diaminoanisole. Inducers of P-450 such as phenobarbital increased somewhat the levels of other metabolites. Thus, there were quantitative but not qualitative differences in the metabolic pathway depending on the inducers used. These studies will eventually afford insight into which forms of the activating enzyme are involved in the various metabolic reactions.

Studies on separating the water soluble metabolites of 2,4-diaminoanisole were begun. As with the organic solvent extractable metabolites, there were quantitative but not qualitative differences in the excretion pattern after treatment with various inducers of P-450. Identification of the water soluble metabolites, which account for a large proportion of a dose of diaminoanisole, will be important in identifying detoxification pathways. A synthesis of an N-hydroxy derivative of 2,4-diaminoanisole has been accomplished which will facilitate further investigations on the metabolism of this compound.

With the dyestuff intermediate 2-aminoanthraquinone it was found that neither the parent compound nor its major metabolites were mutagenic in the Ames Salmonella system. A very small contaminant of 2-AAQ, namely 1,2-diaminoanthraquinone, was found to be very mutagenic in the Ames system indicating that probably two amino groups are necessary for potent mutagenic activity.

Morphological studies with 2,4-diaminoanisole revealed that a goitrogenic effect was noted after only a few weeks of feeding the dye. The noncarcinogenic analog of 2,4-diaminoanisole, m-phenylenediamine, was able to inhibit some of the deleterious morphological effects of 2,4-diaminoanisole on the thyroid and pituitary gland.

It was found that administration of phenobarbital or indole to animals increased the activity of diethylnitrosamine deethylase but the effects on dimethylnitrosamine demethylase were variable. The differential effects of n-butanol and metyrapone on the microsomal diethylnitrosamine deethylase and dimethylnitrosamine demethylase were investigated.

It was found that an endocrine component seemed to be involved in the development of kidney tumors in rats that were administered azoxymethane. These studies are under further investigation.

Electron microscopic investigations on rat hepatocytes which had been transformed by treatment in vitro with nitrosomethylurea, as compared with untreated hepatocytes, showed significant differences between the untreated and transformed cells. These differences could not be detected by ordinary light microscopy. Furthermore, there were marked differences in the catalytic behavior of the lactate dehydrogenase from the control and transformed cells. The transformed cells had a higher initial rate of reaction and higher steady state rate than control cells. Furthermore, the LDH from these cells was unaffected by the Strandford-Clayson inhibitor. Gel electrophoresis also showed that there were no differences in the isozyme components. The proportions of the components in the transformed cells differed from that in the controls.

Analytical Chemistry Section - *"Conducts independent research on the organic, analytical, and biological chemistry of nitrosamines and related compounds and collaborates with other members of the Carcinogenesis staff in matters requiring chemical expertise, especially in problems of identifying non-polymeric organic molecules."* Activities for the past year have focussed on two main areas:

N-Nitroso Compounds. (a) Analysis of rat diets containing added nitrite gave responses consistent with the presence of ppm concentrations of "total nitrosamines" possibly formed by interaction of the nitrite with dietary constituents; efforts are underway to confirm this conclusion by isolating and identifying the individual compounds contributing to the observed responses. (b) The possible role of nitric oxide in environmental nitrosamine formation continues to be studied, with particular emphasis on the chemistry of "Drago complexes" formed from amines and NO. (c) Nitric oxide-oxygen mixtures (or N_2O_3) have been used to N-nitrosate thialdine, a foodstuff constituent which cannot be converted to the N-nitroso derivative using classical nitrosating conditions (nitrite plus mineral acid). (d) 1,2-Dimethylhydrazine (DMH) containing fully deuterated methyl groups has been synthesized for studies of the deuterium isotope effect on the colon carcinogenicity of DMH. (e) Circular dichroism studies on the nitrosamino chromophore thus far have failed to yield a fully general $n \rightarrow \pi^*$ sector rule, but the data suggest that the $\pi \rightarrow \pi^*$ transition may be reliably used for configurational assignments; some evidence for intramolecular hydrogen-bonding has been found in these studies.

Mass Spectrometry. The mass spectrometry laboratory carries out independent research and, in addition, collaborates with other investigators on carcinogenesis related problems in which mass spectrometry can be employed as an analytical tool. (a) Most carcinogenic N-nitrosodialkylamines are well suited for identification by mass spectrometry, which is still the technique of choice for positively confirming suspected nitrosamine contamination in the environment. The electron impact induced fragmentation behavior of dialkylnitrosamines and their C-α-acetoxy analogs is being studied, using deuterium labelled analogs. (b) Spectral studies are continuing on the acetonides of the isomeric 7,8- and 9,10-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrenes, which exhibit distinct mass spectral fragmentation patterns. Also in the polycyclic aromatic hydrocarbon area, high resolution studies on the deoxynucleoside conjugates of 7,12-dimethylbenz[a]anthracene are underway, with the aim of establishing the detailed structures. (c) In supporting projects within the Laboratory, spectral measurements on a number of synthetic intermediates and on metabolites from 2,4-diaminotoluene and 2,4-diaminoanisole have facilitated chemical structure determinations in this series. (d) Efforts are being continued in the analysis of bioactive materials that may play a role in cancer cause and prevention efforts. Collaborative studies aimed at developing more effective chemopreventives in the retinoid series have allowed identification of several new metabolites of retinoic acid. Also, ^{31}P NMR studies are aimed at clarifying the biochemical significance of retinyl phosphate and its conjugates.

Nutrition and Metabolism Section - *"Plans, develops and conducts research on (1) the effects of dietary constituents on the activities of various classes of chemical carcinogens; (2) the metabolism of dietary components known to modify the carcinogenic process; and (3) the correlation of chemical and toxicological data for the identification of chemical carcinogens."* The section has focussed its interest on the role of the lipotropes methionine, choline, vitamin B_{12} and folic acid in chemical carcinogenesis. The growth constants in methionine-deficient, homocysteine-supplemented medium of rat liver epithelial cells were shown to be a linear function of their intra-

cellular levels of methyltetrahydrofolate:homocysteine methyltransferase. No significant differences were observed between the growth constants of transformed cells and those of normal cells in the homocysteine-supplemented medium. The growth constants of transformed cells in complete medium, however, were invariably greater than those of normal cells. Thus the great drop in growth rates suffered by transformed cells placed in homocysteine-supplemented media was due more to their elevated growth rates in the presence of methionine rather than to an abnormal utilization of the amino acid. Methylcobalamin stimulated the growth rates of two transformed lines on homocysteine-containing medium. A thin-layer chromatographic method has been developed for the isolation and quantitative determination of S-adenosylmethionine and S-adenosylethionine in mammalian liver. When rats and hamsters were fed carcinogenic levels of ethionine in the diet, the highest hepatic levels of S-adenosylethionine were observed in the rats. Previous studies have shown that ethionine is tumorigenic to the rat, but not to the hamster. Studies are currently underway comparing the carcinogenicity of ethionine in rats, hamsters and three strains of mice. The levels of ornithine decarboxylase (ODC), a marker of tumor promotion and a precursor enzyme of polyamine biosynthesis, are increased approximately 10-fold in the livers of rats fed a diet containing 0.3% ethionine or devoid of methionine and choline. Although the hepatic levels of S-adenosylmethionine decarboxylase are increased in rats fed the methyl-deficient diet, they are decreased in rats fed ethionine. The section's research activities have also included an examination of species, sex, and strain sensitivities to the formation and inhibition of tumors, and investigations of structure-activity relations in chemical mutagenesis and carcinogenesis. A linear correlation was observed between the increase in lung tumors per mouse produced by a series of alkyl halides and the logarithm of the product of mutagenic activities of the same compounds towards Salmonella TA100 times their maximum tolerated dose in mice.

The production of lung adenomas in strain A mice by nickel acetate and lead subacetate was inhibited by the simultaneous administration of calcium and magnesium acetates. Similarly, calcium inhibited the block by cadmium of the thymidine incorporation into the DNA of liver cells. The possible antagonism between divalent metal carcinogens and the essential divalent cations are being extended to include cell transformation with nickel and testicular carcinogenesis with cadmium.

The role of sex hormones as potential promoters is being investigated. The administration of estradiol to male rats leads to increased renal levels of ODC and S-adenosylmethionine decarboxylase. The induction of renal ODC by estradiol and its analogues is proportional to the in vitro binding by each of the compounds to renal estradiol receptor sites. Chronic treatment of hamsters with estradiol led to increased renal levels of ODC, S-adenosylmethionine decarboxylase, putrescine and spermidine and to the production of kidney adenocarcinomas.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04493-03-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Physico-chemical Studies of Differences between Normal and Tumor-Producing Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.1	1.4

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Physico-chemical studies of differences between normal and tumor-producing cells include: 1) The evaluation of morphological differences, by electron microscopy and scanning electron microscopy, between normal hepatocytes and those which are transformed through exposure to chemical carcinogens in vitro. 2) Comparison of kinetic behavior of lactate dehydrogenase from these two cell lines including application of MLAB On-Line Modelling System for the analysis of data collected in the DASAR System (AMINCO, Silver Spring, MD). 3) Demonstration of qualitatively different response of LDH from two hepatocyte lines and LDH-1 purified from red cells when activity is measured with purified NADH vs commercial preparations of NADH. 4) Development of methods to stabilize the enzymes extracted from the hepatocyte lines. 5) Electrophoretic analysis of these LDH preparations using isoelectric focussing techniques and gel electrophoresis. 6) Efforts to purify the LDH for the two hepatocyte lines by column chromatography and HPLC. 7) Human genetic studies: identification of genetic differences in LDH from a patient with a history of spontaneous abortions followed by identification of the chromosome defect.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Rat hepatocytes, grown in culture free of fibroblasts, and exposed to nitrosomethylurea, produce carcinomas in vivo. During growth in vitro, the chemically transformed cells are not easily distinguished from the control cells by light microscopy, thus hampering the identification of transformation following exposure to chemical carcinogens. Since most human cancers are derived from epithelial cells, the hepatic cell system is a particularly good model for the study of the transformation process. These cells provide a microsomal enzyme system for the oxidation of chemical carcinogens and serve as a target cell for transformation in vitro. In vivo, the processed carcinogen is generally carried to other organs of the body through the large blood supply to the liver and tumors are less frequently found in situ. The long-range objective of these studies is to develop a body of information on the transformation process in the epithelial system.

The immediate objective is to develop more effective methods for the identification of morphological and molecular differences between control and carcinogen-treated hepatocytes. To learn more of the ultrastructural differences between control and transformed hepatocytes, the cells are under investigation by electron microscopy, with special methods developed to preserve the normal intercellular associations characteristic of epithelial cells. Simultaneously, efforts are underway to identify molecular differences in LDH obtained from both types of hepatocytes because of the observation that transformed hepatocytes secrete more acid into the medium during growth.

Additionally, studies begun at the Salk Institute are continuing on: a) investigation of the structure of the nucleoside cyclocytidine (2,2'-anhydro-1-(beta-D-arabinofuranosyl)cytosine), through NMR analysis in an effort to understand its role in DNA metabolism, specifically for studies on DNA damage and repair; b) use of growth factors extracted from serum with organic solvents in an effort to differentiate normal human fibroblasts and those from potential genetic carriers of Gardners' syndrome, a disease which leads to skin and colon cancer.

Methods Employed: A number of physico-chemical methods are used in these studies of normal and transformed hepatocytes: 1) Morphological differences are under investigation by electron microscopy. 2) Differences in the catalytic sites of LDH in cell extracts are studied with the AMINCO-Morrow Stopped Flow DASAR System. The data collected during rapid-reaction assays are analyzed by computer-aided iterations using the MLAB On-Line Modelling System developed by the staff of DCRT, NIH. 3) Electrophoretic separations of the LDH isoenzymes in the two cell lines serve to identify additional structural differences. 4) NMR analysis is being used to determine the structure of cyclocytidine.

Major Findings: At confluence, control hepatocytes, when fixed in situ, showed a nucleus with condensed chromatin and heavy margination at the nuclear membrane. Cytoplasm is half-filled with free polyribosomes, half

rough endoplasmic reticulum (RER). Golgi apparatus is apparent. Mitochondria are somewhat pleomorphic with complete cristae and matrix, underlying the RER. Lysosomes and microbodies are seen occasionally. Plasma membranes have a very irregular surface with many microvilli, coated and pinocytotic vesicles and fibronectin. Transformed cells show dispersed chromatin and thin margination at the nuclear membrane. The cytoplasm has almost no RER, but is filled with dispersed polyribosomes. Mitochondria are fewer than in control cells and have incomplete membranes and matrix, not associated with RER. Plasma membranes are linear in appearance, show only occasional microvilli along about 20% of the surface. These cells lack coated vesicles, pinocytotic vesicles and fibronectin. Further differences were observed 48 hours later. Removal of the cells from the growing surface and fixation in a pellet led to signs of injury, especially in the control cells, and these cells then lost the normal appearance and assumed that of "blast-like" transformed cells.

Marked differences in the catalytic behavior of LDH from control and transformed hepatocytes were observed. Transformed cells have both a much higher initial rate of reaction and a higher steady state rate than control cells. Furthermore, the reaction in transformed cells was comparatively insensitive to product inhibition.

Studies with purified and commercial NADH (still containing inhibitors of the Strandförd-Clayson type) showed that the LDH from transformed cells was almost unaffected by the Strandförd-Clayson inhibitor in contrast to two LDH preparations from normal cells. These results also suggest changes in the catalytic site of the enzyme with transformation.

Gel electrophoresis shows that there are no differences in the isozyme composition: both LDH preparations are LDH-5. Isoelectric focussing studies indicate that both preparations of LDH have six molecular substituents. Five of these show acidic isoelectric points and one component precipitates at pH 9. The important difference is that the proportion of each of the six components in the transformed cell differs from the control, some are increased and some are decreased.

Studies on serum from a patient with a suspected LDH abnormality showed an increase in LDH to above normal levels and a difference in kinetic behavior. Chromosomal analysis revealed the loss of a fragment from chromosome 12, a fragment now shown to be associated with genes controlling LDH, glyceraldehyde-3-phosphate dehydrogenase, and triose phosphate isomerase, all enzymes associated with the glycolytic pathway.

Significance to Biomedical Research and the Program of the Institute: Both morphological and molecular differences are being identified in control and transformed hepatocytes. The methods already developed permit more rapid and positive identification of the transformed cell lessening the need for sole reliance on the lengthy practice of evaluating tumor growth in vivo. The findings are consistent with those identified in neoplastic cells as a shift towards aerobic glycolysis, provide further support for the use of

these epithelial cells as a model system to study individual steps in cell transformation following exposure to chemical carcinogens. This system also offers the opportunity to study carcinogen processing and cellular transformation in a single mammalian cell type.

Structural information pertaining to cyclocytidine may suggest more effective use as a chemotherapeutic agent.

Proposed Course of Project: Further studies are planned to identify molecular differences in LDH and other enzymes in control and transformed hepatocytes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04510-07-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Environmental and Genetic Factors in Digestive System Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard S. Yamamoto	Research Chemist	LCM NCI
OTHER:	Hajime Nawata	Visiting Associate	LCM NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.8	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Female rats are resistant to 1,2-dimethylhydrazine (DMH) carcinogenesis, but not to azoxymethane (AOM) carcinogenesis. AOM was found to produce kidney tumors in female rats, while similar conditions failed to produce kidney tumors in males.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To determine interrelationships of environmental and genetic factors in the development of gastrointestinal tract carcinogenesis, particularly the role of hormones and dietary factors.

Methods Employed: Various strains of rats and mice are compared for their susceptibility to DMH and AOM carcinogenesis. Tumor development, metabolism and other physiological changes are observed.

Major Findings: The response to dimethylhydrazine (DMH) carcinogenesis is quite different between different strains of rats and mice. Kidney tumors developed in females which survived a longer time. Therefore older Fischer rats were administered azoxymethane (AOM) and were kept alive for periods of 12 to 14 months longer. Despite this extended survival time female rats developed kidney tumors and endocrine tumors but the male developed only endocrine tumors. Kidney tumors seemed to be peculiar to female rats which received AOM. Therefore endocrine components may be involved. To investigate this factor DMH was therefore administered to female rats which had been injected with testosterone or estradiol within a 24 hour period after birth. These hormones tend to interfere with the hypothalamic or pituitary control of the endocrine glands. Under these conditions DMH did induce a few intestinal tumors in the controls, but there were many more intestinal tumors in those treated with the hormones. All the rats which received testosterone had kidney tumors, and some rats had them in both kidneys. Thus, the endocrine balance had a role in the kidney tumor formation with DMH or AOM.

Significance to Biomedical Research and the Program of the Institute: The high morbidity and mortality from gastrointestinal cancer is a major health problem in the United States. Men are more susceptible than women when considered from the age and per 100,000 to colon cancer in most of the countries in the world. There seem to be a major hormone involved in colon cancer development.

Proposed Course of Project: Further studies of the effect of dietary and hormonal factors will be made in conjunction with compounds known to produce gastrointestinal tract cancer. Resistant versus susceptible strains and species will be used to study the metabolic activation and tumorigenicity of colon carcinogens.

Publications

Yamamoto, R. S., Frankel, H. H., and Weisburger E. K.: Effect of phenacetin and N-alkylacetanilides on N-2-fluorenylacetamide carcinogenesis. Cancer Ltrs. 8: 183-188, 1979.

Yamamoto, R. S.: Colon carcinogenesis. In Sugimura, T. and Yamamura, Y. (Eds.): Gann (Cancer). Tokyo, Japan, Iwanami Shoten, 1978, pp. 120-126.

Yamamoto, R. S.: Effect of vitamin B_{12}-deficiency in colon carcinogenesis. Proc. Soc. Exp. Biol. Med. 163: 350-353, 1980

Nishinaga, K. and Yamamoto, R. S.: Electrophoretic characterization of mouse liver polyribosomes. Anal. Biochem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04542-08-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chemistry of N-Nitroso Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. Keefer	Chief, Analytical Chemistry Section	LCM NCI
OTHER:	P. Roller	Chemist	LCM NCI
	J. Fanning	Chemist	LCM NCI
	P. Hillery	Guest Worker	LCM NCI
	T. Hansen	Staff Fellow	LCM NCI

COOPERATING UNITS (if any) Drs. W. Gaffield and R. Lundin, Western Regional Research Laboratory, USDA; Drs. C. Walters and R. Hart, British Food Manufacturing Industries Research Association; Dr. P. Newberne, Massachusetts Institute of Technology; Drs. P. Swann and R. Mace, The Middlesex Hospital Medical School

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Analytical Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Data concerning the chemical and physical properties of the carcinogenic N-nitroso compounds will be collected. Specific emphases will include: a) the preparation of novel nitrosamines and their derivatives for chemical and biological studies; b) the biological chemistry of nitrosamines, both in vivo and in microsomal systems, with special attention to the rate modifying effects of deuterium substitution; c) mechanistic studies of N-nitrosation reactions of environmental interest, particularly those promoted by electrophilic species, including transition metal complexes. Possible implications of this work with respect to the overall goal of human cancer prevention will be considered.

by subjecting an isomer of dimethylnitrosamine (azoxymethane) to base catalyzed exchange with heavy water, followed by addition of aluminum foil to reduce the azoxy compound to the hydrazine; this material will be sent to Dr. V. Turusov for a test of its carcinogenic potency compared with that of the undeuterated parent compound.

In the physical chemical component of this project, a series of heterocyclic nitrosamines has been studied in collaboration with Drs. W. Gaffield and R. Lundin of the U.S. Department of Agriculture using nuclear magnetic resonance, infrared, and circular dichroism techniques. Data suggesting intramolecular hydrogen bonding between the nitroso nitrogen and a hydroxymethyl or carboxyl function at the 2-position have been found. Unfortunately, the major purpose of this collaboration, to develop a definitive and generally applicable sector rule for relating circular dichroism data to chemical structure, has not been achieved. It appears that the $n \rightarrow \pi^*$ transition of the nitrosamino chromophore may not be amenable to such a conclusion. However, it seems possible based on our data that the $\pi \rightarrow \pi^*$ transition might be well suited to such stereochemical studies, with all compounds we have investigated giving negative Cotton effects for the enantiomer having the L-proline configuration.

Significance to Biomedical Research and the Program of the Institute: Carcinogenic nitrosamines have been detected in an increasing array of environmental sources. We plan to continue studying their mechanisms of formation under environmental conditions with the hope of learning enough about these pathways to prevent them from occurring. Hopefully, human contact with carcinogenic nitrosamines can be reduced in this way. However, since such contact probably cannot be completely eliminated, we also plan to study the chemistry of the nitrosamine-organism interaction with the aim of accumulating enough knowledge to permit development of strategies for preventing tumor formation after exposure has occurred.

Publications

Keefer, L. K.: α-Amino nitrite esters and their analogues: Possible reactive intermediates in N-nitrosamine formation? In Anselme, J.-P. (Ed.): N-Nitrosamines. ACS Symposium Series No. 101, Washington, D.C., American Chemical Society, 1979, pp. 91-108.

Kleihues, P., Doerjer, G., Keefer, L. K., Rice, J. M., Roller, P. P. and Hodgson, R. M.: Correlation of DNA methylation by methyl(acetoxymethyl)-nitrosamine with organ-specific carcinogenicity in rats. Cancer Res. 39: 5136-5140, 1979.

Emmett, G. C., Michejda, C. J., Sansone, E. B. and Keefer, L. K.: Limitations of photodegradation in the decontamination and disposal of chemical carcinogens. In Walters, D. (Ed.): Safe Handling of Carcinogens, Mutagens, Teratogens and Highly Toxic Substances, Chapter 28, Vol. 2. Ann Arbor, MI, Ann Arbor Press Science Publishers, Inc., 1980, pp. 535-553.

Roller, P. P., Keefer, L. K. and Slavin, B. W.: Inhibitory agents and chemical mechanisms in the dihalomethane mediated nitrosation of amines with solid nitrite. In proceedings of Sixth Meeting on Analysis and Formation of N-Nitroso Compounds. Lyon, IARC Scientific Publications, 1980, (in press).

Walters, C. L., Hart, R. J., Keefer, L. K. and Newberne, P. M.: The sequential determination of nitrite, N-nitroso compounds and nitrate and its application. In proceedings of Sixth Meeting on Analysis and Formation of N-Nitroso Compounds. Lyon, IARC Scientific Publications, 1980, (in press).

Croisy, A. F., Fanning, J. C., Keefer, L. K., Slavin, B. W. and Uhm, S.-J.: Metal complexes as promoters of N-nitrosation reactions: A progress report. In proceedings of Sixth Meeting on Analysis and Formation of N-Nitroso Compounds. Lyon, IARC Scientific Publications, 1980, (in press).

Project Description

Objectives: (1) To study mechanisms of metabolism and carcinogenesis by N-nitroso compounds using deuterium labelled substrates. (2) To synthesize novel N-nitroso compounds, including possible metabolites of certain carcinogens, for physical, chemical, and biological investigations. (3) To study mechanisms by which metal ions and other electrophilic species might promote the formation of N-nitroso compounds in the environment.

Methods Employed: The standard methods of synthetic and mechanistic chemistry have been used in these investigations. In addition, a 200 MHz nuclear magnetic resonance spectrometer with superconducting magnet and complete data system has been installed. This resource is expected to help us greatly in studying chemical structure and dynamics.

Primary attention has continued to focus on the chemistry of nitrosamine formation under conditions of possible interest in developing cancer prevention strategies. In collaboration with scientists at the British Food Manufacturing Industries Research Association (BFMIRA) and the Massachusetts Institute of Technology, rat diets to which nitrite had been added were studied for possible accumulation of nitrosamines through the interaction of dietary components with the added nitrite. "Total nitrosamine" concentrations in these diets were found to be in the ppm range, suggesting that N-nitroso compounds presently impossible to determine analytically might be forming in such media and complcating the interpretation of feeding studies in which nitrite is added to animal diets. However, the BFMIRA method has not yet been validated, and it is possible that much of the observed response is due to false positives, i.e. to compounds which do not contain the N-nitroso group. In the final phase of this work, we plan to subject our analyses to detailed comparisons with analytical data obtained by other investigators using generally accepted assay methods. We shall also attempt to isolate one or more of the major components responsible for the observed response to the BFMIRA technique, then determine by standard structural elucidation methods whether they are N-nitroso compounds or not.

We have continued to explore the suitability of nitric oxide as a nitrosating agent. In contrast to previous expectations, nitric oxide by itself proved not to be the reagent of choice for N-nitrosation of thialdine, a sulfur-nitrogen heterocycle whose N-nitroso derivative could not be prepared using the classical mineral acid/aqueous nitrite procedure. The N-nitroso thialdine has now been efficiently prepared using N_2O_3 or butyl nitrite-acetic acid in an organic solvent. The reactivity of nitric oxide as a possible N-nitrosating agent will be further explored using other amines previously reported by Drago and coworkers to form nitrosamines via complexes of the formula $R_2N\text{-}NO\text{-}NO^-$ $R_2NH_2^+$.

In our synthetic chemistry effort, 3,3,4,4-tetrafluoropyrrolidine has been prepared and converted to its N-nitroso derivative; we plan to compare the reactivity of this weak base toward solid sodium nitrite in methylene chloride solution with that of pyrrolidine. In addition, the colon carcinogen, 1,2-dimethylhydrazine, has been prepared with deuterium label in both methyl groups

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04580-06-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Role of Lipotropes in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lionel A. Poirier	Chief, Nutrition & Metabolism Sec.	LCM NCI
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	Camille Hyde	Staff Fellow	LCM NCI
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	Nina V. Myasishcheva	Guest Worker	

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.6	2.7	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms behind the alteration of chemical carcinogenesis by the dietary lipotropes, choline, methionine, folic acid and vitamin B_{12} have been studied. The metabolism and carcinogenic activity of ethionine in different species is being compared. Ornithine decarboxylase, a marker of tumor promotion and a precursor enzyme for the polyamines, is used to study the possible role of methyl insufficiency in tumor promotion. The great drop in growth rates of transformed liver epithelial cells placed in methionine-deficient, homocysteine-supplemented medium is due to their elevated growth rates in complete medium, rather than to an inability to synthesize methionine.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The dietary lipotropes methionine, choline, and vitamin B_{12} significantly modify the production of tumors by certain chemical carcinogens. The extent of and the mechanisms behind these effects are being investigated. The effects of the lipotropes on chemical carcinogenesis are under investigation. In addition, the mutual metabolic interactions of the lipotropes and the chemical carcinogens are to be determined.

Methods Employed: The carcinogenic activities of several standard carcinogens were compared in rats fed defined diets. S-Adenosylmethionine and S-adenosylethionine are determined using isotope-dilution techniques and thin layer chromatography followed by spectrophotometric assay. The nutritional requirements of normal and transformed liver epithelial cells were determined in tissue culture systems developed in this laboratory.

Major Findings: The growth constants of both normal and transformed hepatocytes on methionine-deficient and homocysteine-supplemented medium were found to be directly proportional to their intracellular levels of the enzyme N^5-methyltetrahydrofolate homocysteinyl:methyltransferase, when assayed in the absence of exogenous vitamin B_{12}. Methylcobalamin stimulated the growth of 2 transformed cell lines on the methionine-deficient, homocysteine-supplemented medium. The chronic administration of methyl-deficient, amino acid-defined diets led to increased hepatic levels of ornithine decarboxylase (a marker of tumor promotion), S-adenosylmethionine decarboxylase, and thymidine kinase; the levels of these enzymes tended to be inversely correlated to the methyl content of the diet. Ethionine, which is carcinogenic to rat liver, but noncarcinogenic in hamsters, formed higher hepatic levels of S-adenosylethionine in rats than in hamsters. The chronic administration of ethionine to rats increased the hepatic levels of ornithine decarboxylase but suppressed S-adenosylmethionine decarboxylase. Hepatic ornithine and S-adenosylmethionine decarboxylases were induced by dietary deficiencies of methionine, choline, and vitamin B_{12}.

Significance to Biomedical Research and the Program of the Institute: One of the basic aims of the National Cancer Institute is the prevention of cancer by a delineation of the mechanism by which carcinogens induce tumors. The aim of these studies is to amplify the inhibitory effects of excesses or deficiencies of lipotropes on the carcinogenic process by understanding the mechanism by which they exert these effects.

Proposed Course of Project: Carcinogenesis by standard carcinogens in animals fed chemically-defined diets will be determined. The carcinogenicity and metabolism of ethionine in different species will be completed. The effects of methionine deficiency on tumor promotion will be determined. The metabolism of selenium and the carcinogenicity of its metabolites will be examined.

Publications

Chauveau, J., Decloitre, F. and Mikol, Y. B.: Intervention des Polluants de L'Environnement dans la Cancerogenese Chimique: Action du Lindane et du captane sur la Metabolisation du Dimethylaminoazobenzene et du Benzo(a)pyrene. Journees du Comite Scientifique Contamination des Chaines Biologiques. Ministere de l'Environement et du Cadre de Vie. Paris (February, 1980).

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Hyde, C. L., Rusten, R., and Poirier, L. A.: A thin-layer chromatographic method for the quantitative separation and estimation of S-adenosylmethionine and S-adenosylethionine in rat liver. Anal. Biochem. (in press).

Poirier, L. A. and Wilson, M. J.: The elevated requirement for methionine by transformed rat liver epithelial cells in vitro. Proc. NY Acad. Sci. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04581-05-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Analytical Applications of Mass Spectrometry to Carcinogenesis Problems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	L. K. Keefer	Chief, Analytical Chemistry Section	LCM NCI
	J. R. Miller	Chemist	LCM NCI
	B. W. Slavin	Physical Science Aide	LCM NCI
	E. K. Weisburger	Chief	LCM NCI
	P. H. Grantham	Chemist	LCM NCI
	T. Benjamin	Chemist	LCM NCI
	M. B. Sporn	Chief	LCP NCI
	C. A. Frolik	Chemist	LCP NCI
	H. V. Gelboin	Chief	LMC NCI
	R. M. Angeles	Staff Fellow	LMC NCI
	L. M. DeLuca	Chief, Differentiation Control	LEP NCI

COOPERATING UNITS (if any)
S. K. Yang, USUHS, Bethesda, MD; C. J. Michejda, FCRC, Frederick, MD; A. M. Jeffrey, Columbia U., New York, NY.

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Analytical Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	0.8	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The laboratory is involved in carrying out independent research, as well as participating in collaborative projects on carcinogenesis related problems, where mass spectrometry can be used as an analytical tool to determine the structure or confirm the identity of non-polymeric organic molecules of interest. Studies include: (1) the identification of carcinogen metabolites in activation and metabolism studies; (2) the identification of naturally occurring potential carcinogens in the environment; (3) elucidation of mass spectral fragmentation mechanisms; (4) development of methods for derivatization and analysis of carcinogens; (5) analysis of bioactive materials that may play a role in cancer causation and prevention mechanisms, such as vitamin A derivatives and substances with immunological properties; (6) application of nuclear magnetic resonance spectroscopy to structural problems.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: (1) To study in some detail the mass spectra of carcinogens such as N-nitroso compounds, aromatic hydrocarbons and aromatic amines, as well as some of their possible metabolites, with the aim of applying this knowledge to develop appropriate analytical methods. (2) To apply the mass spectrometry method for the analysis and identification of metabolites in carcinogen activation and metabolism studies and to elucidate the enzymatic mechanisms of such transformations using deuterium labelled substrates. (3) To elucidate the chemical structure of certain natural bioactive materials, or of their synthetic analogs, that may play a role in cancer causation and prevention mechanisms.

Method Employed: An in-house high resolution double focusing mass spectrometer system was used, interfaced with a gas chromatograph, and run in the electron impact ionization mode. A number of synthetic and metabolic samples were also prepurified by collaborators using high pressure liquid chromatography. In some cases other suitable chemical and spectral characterizations were necessary. Increasing use was made of the Section's high resolution superconducting-magnet nuclear magnetic resonance (NMR) spectrometer giving important complementary information to mass spectrometry in structural and other studies. The NMR technique is also being applied to the analysis of materials that are thermally unstable or non-volatile, i.e. too polar to be vaporized for mass spectral measurements.

Major Findings: The mass spectrometry program operates at 3 levels of effort: first, we engage in independent projects on our own initiative; second, the facility supports the chemical studies related to nitrosamines and metal complexes within the Analytical Chemistry Section; third, the group engages in collaborative studies on projects of mutual interest with investigators within the Laboratory of Carcinogen Metabolism and elsewhere within the Institute.

Independent studies are continuing to examine in detail the mass spectral behavior of specific classes of compounds, particularly the aliphatic dialkylnitrosamines and α-acetoxydialkylnitrosamines, so that the information gained can be utilized in structural confirmation of known compounds or in determination of related, yet unknown materials. The mass spectra of aliphatic nitrosamines revealed a rich variety of molecular rearrangements and fragmentations, and indicated also the strong directing effect of the nitrosamino moiety. Specifically, the electron impact induced hydroxyl radical loss in the mass spectra of the N-methyl butyl-, hexyl-, and octyl-nitrosamines is being studied in detail using high resolution techniques and information gained from the fate of the deuterium atoms in the mass spectra of deuterium labelled analogs.

A number of recent studies indicate that the activated carcinogenic metabolite of benzo(a)pyrene (B(a)P) is the 7,8-trans-dihydrodiol-9,10-epoxide derivative. Carbon 10 of this molecule possesses a special chemical reactivity toward nucleophiles. According to our earlier studies the hydroxyl group at C-10 of certain isomers of the 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydro-B(a)P is also susceptible to substitution. Current studies reveal that the isomers of the

7,8- and 9,10-diol derivatives of 7,8,9,10-tetrahydro-B(a)P possess very dissimilar reactivities toward isopropylidene (acetonide) derivative formation and the apparent anomalous behavior of the 9,10-cis and -trans diols is attributable to the special lability of substituents on carbon 10. The mass spectra of the isomeric 7,8- and 9,10-isopropylidene derivatives thus formed exhibit distinct mass spectral fragmentation patterns. These mechanisms are being investigated using ^{18}O and deuterium labelled analogs.

Several collaborative projects are concerned with investigations of bioactive materials that may play a role in cancer prevention mechanisms. In joint studies with Frolik and Sporn detailed mass spectral and NMR analysis demonstrated the in vivo cis-trans isomerization and oxidation of retinoic acid in hamster and in vitro in hamster liver. Separate recently initiated ^{31}P NMR studies with DeLuca's group are aimed at delineating the biochemical pathways and the role of retinyl phosphate and its sugar conjugates in biological systems.

In collaborative studies within the Laboratory, in vivo metabolic studies of 2,4-toluenediamine and 2,4-diaminoanisole in the rat are progressing and recent efforts are concerned with mass spectral and NMR characterization of the water soluble metabolites. Efforts are also continued in determining the point of linkage of DMBA to DNA in in vitro metabolic studies with 3,4-dihydroxy-DMBA using high resolution mass spectral methods in collaborative studies with the Columbia University group of Jeffrey and Weinstein. In collaborative work with Michejda and coworkers at FCRC, quantitation using our gas chromatography-mass spectrometry system and deuterium labelled substrates indicates, that the in vitro microsomal oxidation system that metabolizes DMN, also methylates overwhelmingly the particular electrophile trapping agent used, 3,4-dichlorothiophenol, independently of the carcinogen's alkylating action. The biochemical significance of this latter finding awaits evaluation.

Significance to Biomedical Research and the Program of the Institute: The understanding of the mass spectral behavior of specific classes of organic compounds is a prerequisite to the interpretation of spectra and to the structural determination of a number of biologically important compounds. Toward this goal detailed spectral studies on known N-nitroso compounds and polycyclic aromatic compounds have been valuable. Structural studies on metabolites of aromatic amines and of polycyclic aromatic hydrocarbons have given insight into the pathways of carcinogen degradation or activation. Identification of carcinogens or of potential carcinogens in man's immediate environment or food staples is important in developing prevention methods.

Proposed Course of Project: The studies described above will be completed and published. A number of metabolic studies are continuing and their significance to cancer causation will be evaluated.

Publications

Woolard, F. X., Moore, R. E. and Roller, P. P.: Halogenated acetic and acrylic acids from the red alga Asparagopsis taxiformis. Phytochem. 18: 617-620, 1979.

Goren, M. B., Brokl, O. and Roller, P.: Cord factor (trehalose-6,6'-dimycolate) of in vivo-derived Mycobacterium lepraemurium. Biochim. Biophys. Acta 574: 70-78, 1979.

Borzsonyi, M., Ferencz, A., Surjan, A., Pinter, A., Nadasdi, I., Roller, P. P., Torok, G. and Kiss, K.: Mutagenicity of N-nitrosopyridinol carbamate with the Salmonella/mammalian microsome test. Cancer Lett. 7: 135-139, 1979.

Grantham, P. H., Benjamin, T., Tahan, L. C., Roller, P. P., Miller, J. R. and Weisburger, E. K.: Metabolism of the dyestuff intermediate 2,4-diaminoanisole in the rat. Xenobiotica 9: 333-341, 1979.

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Berman, J. J., Rice, J. M., Wenk, M. L. and Roller, P. P.: Dependence of tumor spectrum on route of administration in Sprague-Dawley rats as a result of single or multiple injections of methyl(acetoxymethyl)nitrosamine. J. Natl. Cancer Inst. 63: 93-100, 1979.

Berman, J. J., Rice, J. M., Wenk, M. L. and Roller, P. P.: Intestinal tumors induced by a single intraperitoneal injection of methyl(acetoxymethyl)nitrosamine in three strains of rats. Cancer Res. 39: 1462-1466, 1979.

Jeffrey, A. M., Grzeskowiak, K., Weinstein, I. B., Nakanishi, K., Roller, P. and Harvey, R. G.: Benzo(a)pyrene-7,8-dihydrodiol 9,10-oxide adenosine and deoxyadenosine adducts: Structure and stereochemistry. Science 206: 1309-1311, 1979.

Grantham, P. H., Mohan, L., Benjamin, T., Roller, P. P., Miller, J. R., and Weisburger, E. K.: Comparison of the metabolism of 2,4-toluenediamine in rats and mice. J. Environ. Path. Toxic., 3: 149-166, 1979.

Roller, P. P., Keefer, L. K. and Slavin, B. W.: Inhibitory agents and chemical mechanisms in the dihalomethane mediated nitrosation of amines with solid nitrite. In Walker, E. A., Griciute, L., and Borzsonyi, M. (Eds.): Sixth Meeting on Analysis and Formation of N-Nitroso Compounds. Lyon, IARC (in press).

Frolik, C. A. and Roller, P. P.: The role of retinoids (vitamin A and its analogs) in the prevention of epithelial cancer. In Aszalos, A. A. (Ed.): Antitumor Compounds of Natural Origin. West Palm Beach, Florida, CRC Press Inc., Chapter 9, (in press).

Borzsonyi, M., Torok, G., Pinter, A., Nadasdi, L. and Roller, P. P.: Carcinogenic effect of dinitrosopiperazine in adult Swiss mice and after transplacental or lactational exposure. Cancer Res., 1980, (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04582-05-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure Activity Correlations in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lionel A. Poirier	Chief, Nutrition & Metabolism Sec.	LCM NCI
OTHER:	Camille Hyde	Staff Fellow	LCM NCI
	Lawrence Lanier	Biological Laboratory Technician	LCM NCI

COOPERATING UNITS (if any)

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Dr. V. Simmon, SRI International, Menlo Park, California
Dr. M. Wenk, Microbiological Associates, Bethesda, Maryland

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.9	0.6

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mutagenic and carcinogenic activities of previously untested agents were investigated. Salmonella TA100 was used to determine the mutagenic activities of alkyl halides. The ability of alkyl halides to produce lung adenomas in strain A mice was also studied. In general a linear correlation was observed between mutagenicity and carcinogenicity. The antagonism between the essential divalent metals calcium and magnesium and the divalent metal carcinogens, lead, nickel and cadmium are under investigation in metabolic and in carcinogenicity studies. Magnesium and calcium completely prevented lung adenoma production in strain A mice by nickel and lead acetates.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The accumulation of evidence has indicated that the activated form of most, if not all, chemical carcinogens consists of a reactive electrophile. The aim of these studies is to determine the extent to which the electrophilic hypothesis of carcinogenesis may be used to explain the mechanisms of chemical carcinogenesis. Mutagenesis towards microbial systems in vitro is used to obtain rough measure of a compound's ability to produce reactive intermediates. Possible mechanisms by which chemicals of diverse structure exert their carcinogenic activity are proposed from a knowledge of their chemical reactivity, structure, metabolic pathways and mutagenic activity.

Methods Employed: The carcinogenic activities of previously untested compounds are tested by standard protocols, generally by the production of mouse lung adenomas in strain A mice. Alternate carcinogenesis studies are done by long-term feeding, injection or intubation of the suspect compound into rats, followed by examination for tumors at necropsy. Mutagenicity studies are being performed with the Ames assay with Salmonella TA100. Thymidine incorporation into the DNA of rat liver cells is accomplished by standard radioisotopic, cell culture and ultracentrifugation techniques.

Major Findings: The tumorigenic activity of a series of substituted alkyl halides in the mouse lung adenoma test was found to be a linear function of the logarithm of the product of the mutagenicity towards Salmonella TA100 of each of the compounds times its maximal tolerated dose in the mice. Also, the tumorigenic activities of nickel acetate and lead subacetate towards the lungs of strain A mice were completely prevented by the simultaneous administration of calcium acetate and of magnesium acetate. Cadmium chloride inhibited the uptake of thymidine into the DNA of rat liver cells in culture; such inhibition was prevented by increased levels of calcium in the medium.

Significance to Biomedical Research and the Program of the Institute: The aim of these studies is to increase the base of theoretical knowledge by which the potential carcinogenic hazards to man of previously untested carcinogens can be estimated. The evidence accumulated to date indicates that with the alkyl halides biological activity tends to parallel chemical activity and that an antagonism to the divalent cations calcium or magnesium may be part of the mechanism by which the divalent metal carcinogens exert their activity.

Proposed Course of Project: The carcinogenicity of non-alkylating carcinogens will be examined. These include compounds with oxidized electrophilic carbon, arsenic and chromium. Attempts will be made to extend the observed antagonism between carcinogenic divalent metals and calcium and magnesium.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04589-03-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Endocrine Factors in Tumor Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Hajime Nawata	Visiting Associate	LCM NCI
Co-PI:	Dr. Lionel Poirier	Chief, Nutr. & Met. Section	LCM NCI
OTHER:	Dr. Richard Yamamoto	Chemist	LCM NCI

COOPERATING UNITS (if any)

None

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SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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1.0	1.0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of hormones on the induction of ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), and polyamine levels in vivo are being studied. Previous studies have shown that ODC induction may be a marker for tumor promoters. Hormones often increase the biological activities of complete carcinogens. ODC is used to study the degree of biochemical similarity between hormones and promoters. The present studies indicate that estradiol induces renal ODC and SAMDC in both the rat and hamster. The induction of renal ODC in the rat by estrogens is proportional to their binding in vitro to cytoplasmic estradiol binding sites. ODC levels in the ovary and uterus of female rats are inversely correlated with each other. Estrogens as potential promoters will be tested in two-stage initiation-promotion carcinogenesis studies.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The major objective of these studies is to determine whether the enhancement of the activity of certain complete carcinogens by hormones is due to a promoting activity of the hormone.

Previous studies have demonstrated that certain hormones often increase the activity of specific carcinogens towards their target organs. In theory, such effects may be due to (a) altered metabolism, increasing the effective intracellular concentration of ultimate carcinogens, (b) promotion of initiated cells and (c) other incompletely defined co-carcinogenic effects. Previous studies have shown that hormones frequently alter carcinogen metabolism. We are attempting to show that hormones may act as promoters. The induction of ornithine decarboxylase (ODC) is used as marker of promoting-like activity.

Methods Employed: The enzymes ODC and S-adenosylmethionine decarboxylase are assayed by trapping labelled CO_2. Tissue levels of polyamines are determined by thin-layer chromatography and fluorimetric analysis. The enzymes and the polyamines are determined in multiple organs of intact and hormonally-ablated rats and hamsters, which have been treated with hormones.

Major Findings: (a) Testosterone enhances and estradiol inhibits the induction of ODC by growth hormone in the livers of rats. (b) Renal ODC, SAMDC and polyamine levels are increased by estradiol administration. The induction of renal ODC in the rat by estrogen analogues is proportional to their *in vitro* interaction with cytoplasmic estradiol binding sites. (c) There is an inverse correlation between the levels of ODC and SAMDC in the ovary of rats and their corresponding level in the uterus. The observed effects of exogenous hormones on the tissue levels of ODC generally parallel the effects of the same hormones on carcinogenesis.

Significance to Biomedical Research and the Program of the Institute: A major goal of the NCI is to determine the mechanism of action of chemical carcinogens. These studies attempt to clarify the contribution of hormones to the carcinogenic process in man.

Proposed Course of Project: To test the hypothesis that hormones often act as promoters, the following investigations will be pursued: 1) Studies of ODC induction by hormones will be extended and compared to the effects of the same hormones on DNA-synthesis and cell mitosis in the same organs. 2) The predictability of hormonal induction of ODC will be tested by initiation-promotion experiments. Short pulses of complete carcinogens will be used as initiators and hormones as promoters, in those tissues in which they induce ODC.

Publications

Nawata, H., Kato, K., and Ibayashi, H.: Properties of partially purified thymidine kinase in adrenal tissues of hyperplasia, adenomatous hyperplasia, adenoma, and carcinoma of patients with Cushing's Syndrome. Acta Endocrinol. 93: 208-215, 1980.

Nawata, H., Yamamoto, R. S., and Poirier, L. A.: Ornithine decarboxylase induction and polyamine levels in the kidney of estradiol-treated male rats. Life Sci. 26: 689-698, 1980.

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Nawata, H., Sakai, K., Kato, K., and Ibayashi, H.: Pseudohypoparathyroidism with response by Ellsworth-Howard's test following the normal serum Ca level. Folia Endocrin. Jap. (in press).

Nawata, H., Haji, M., Kato, K., and Ibayashi, H.: Testosterone and estrogen receptors of pituitary and hypothalamus in young and old rats. Endocrinology (in press).

Nawata, H., Kato, K. and Ibayashi, H.: Familial SLE with gastric cancer. Clin. Immunol. (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04617-15-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Carcinogen Screening Operations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elizabeth K. Weisburger	Chief	LCM NCI
OTHER:	Timothy Benjamin	Chemist	LCM NCI
	Carolyn A. Brown	Microbiologist	LCM NCI
	Ritva P. Evarts	Veterinary Medical Officer	LCM NCI

COOPERATING UNITS (if any)
Dr. M. B. Shimkin, University of California, La Jolla; Dr. B. Ulland, Hazleton Labs., Virginia; Dr. Donahoe, Microbiological Associates, Bethesda, Md.; and Dr. A. S. Krishna Murthy, Mason Research Institute, Worcester, Mass.

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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2.5	2.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The chronic effects of environmental chemicals or of mixtures of chemicals are determined in suitable animal models. Factors influencing the outcome such as sex, strain, and species of animal are investigated.

Project Description

Objectives: Responsibilities are assumed on the design, performance, and evaluation of studies in either contract or intramural laboratories, on the chronic effect and carcinogenicity of chemicals or of mixtures of chemicals. The aim of these investigations is to gather information of hazards involved in handling certain chemicals or drugs, and also on the mechanisms underlying joint effects in mixtures. Of particular interest are such mixtures which tend to give increased or decreased overall carcinogenic effect. Of concern also is the methodology of carcinogen screening, with emphasis on improvements in sensitivity, speed and economy. Short-term assays are under development, taking into account the need to secure biochemical activation of most environmental agents. Guidelines for assessment of the carcinogenic potential of chemicals are developed, by consideration of their chemical structure, metabolic pathways and mutagenic activity.

Methods Employed: Attempts are made to secure information on chemicals representing the greatest hazard to the largest number of people. Chemicals and drugs are rated as to priority on this basis as well as other criteria such as relationship to known carcinogens, epidemiologic observations, indications of specific toxicity and related factors. Standard protocols as well as newly designed protocols attempting to increase the sensitivity and speed of such tests are utilized. The carcinogenic and mutagenic activities of various compounds are determined in appropriate systems, either directly or after metabolic activation. Exploratory meetings with relevant national and international organizations in industry, in government and in university environments are setting the stage for a concerted development effort in this area.

Major Findings: Industrial or environmental chemicals. Papers are in press or in preparation on the long term effects of various industrial or environmental chemicals such as pesticides, industrial intermediates and dyestuff intermediates.

Significance to Biomedical Research and the Program of the Institute: Many substances in the environment have not yet been evaluated for chronic toxicity and possible carcinogenicity. It is important to secure such information after deliberate and judicious setting of priorities. Cancer in man has been observed in the past as a result of exposure to chemicals subsequently demonstrated to be carcinogenic. The aim of this program is to forestall such unintentional exposures of man which would result in neoplastic disease in the future.

Proposed Course of Project: Continuing efforts are required 1) to assess relative priorities of materials to be tested, 2) to undertake studies on variations in the protocols to simplify or to develop novel test techniques which are more sensitive, more specific and more economic, and 3) to apply such tests to identify carcinogenic hazards in the environment.

Publications

Krishna Murthy, A. S., Russfield, A. B., Hagopian, M., Monson, R., Snell, J. and Weisburger, E. K.: Carcinogenicity and nephrotoxicity of 2-amino-, 1-amino-2-methyl-, and 2-methyl-1-nitroanthraquinone. Toxicol. Ltrs. 4: 71-78, 1979.

Weisburger, E. K.: Carcinogenic agents. In Pradhan, S. N. (Ed.): Textbook of Pharmacology. St. Louis, Missouri, C. V. Mosby Co., (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04618-15-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Endogenous and Exogenous Factors in Chemical Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ritva P. Evarts	Veterinary Medical Officer	LCM NCI
OTHER:	Carolyn A. Brown	Microbiologist	LCM NCI
	Elizabeth K. Weisburger	Chief	LCM NCI

COOPERATING UNITS (if any)

Dr. B. Cockrell, Experimental Pathology Laboratories

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NCI, NIH, Bethesda, Maryland 20205

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4.3	2.3	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects structural alterations may have on the carcinogenicity or toxicity of various compounds are determined. Methods to inhibit the action of known carcinogens by administration of other compounds are devised. The biochemical, morphological, and physiological bases for such inhibitory effects are studied.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Investigations to determine the intimate factors involved in initiation and development of neoplasia are performed. The current aims are 1) to study the overall effect of the hair dye ingredient 2,4-diaminoanisole on the morphology and tumor frequency in different tissues; 2) to determine the function of liver microsomal enzyme systems in carcinogenesis of peripheral tissues, especialy of the kidney and mammary gland; 3) to learn more about the kinetics and characteristics of the enymes involved in the metabolism of dimethylnitrosamine and azo dyes; and 4) to evaluate the effect of mammary gland morphology on the type of tumor produced by chemical carcinogens.

Methods Employed: Evaluation of the effects of dietary and chemical factors on the liver microsomal enzymes was based on both in vivo and in vitro studies of different components of the liver mixed function oxidase system. Liver enzyme activity was monitored after partial hepatectomy or after various dietary or chemical treatments.

Major Findings: (1) 2,4-Diaminoanisole (2,4-DAA). A short term experiment showed that feeding 2,4-DAA for 4 weeks to Fischer female rats revealed a goitrogenic effect due to the feeding of the dye, with prominent pigmentation of the epithelium of the thyroid follicular cells. In the pituitary glands large band staining cells were seen. When the non-carcinogenic analog, m-phenylenediamine (m-PDA), was fed at concentrations of 1000 and 2000 ppm for 4 weeks, only slight hypertrophy of the thyroid gland occurred. Microscopically, the thyroid epithelial was almost normal and no pigmentation was observed. However, many mitotic figures were seen in the thyroid and pituitary gland. When the two compounds were fed together no thyroid pigmentation occurred as with 2,4-DAA alone. Only slight epithelial hypertrophy was seen. Furthermore in the pituitary no pale staining cells were noted. Thus, the m-PDA seemed to inhibit the short term morphological effects of 2,4-DAA on the thyroid and pituitary gland.

(2) Microsomal diethylnitrosamine deethylase (DEN-d) and dimethylnitrosamine demethylase (DMN-d). Administration of phenobarbital or indole to animals increased the activity of DEN-d. On the other hand the activity of DMN-d was inhibited by phenobarbital but increased by administration of indole. Studies with metyrapone which inhibits the beta binding site of cytochrome P-450 showed that it was a relatively potent inhibitor for DEN-d. On the other hand n-butanol which binds to the alpha binding site was a more potent inhibitor for DMN-d. Studies on these molecular effects of the inhibitors are continuing.

Significance to Biomedical Research and the Program of the Institute: The prevention and cure of neoplasia hinges on an understanding of the intimate factors involved in the pathogenesis of cancer. The research performed within the framework of this report aims to produce data which will permit the comprehension of the underlying mechanisms involved in chemical carcinogenesis.

Proposed Course of Project: Emphasis will continue on the function of the liver mixed function oxidase system in peripheral tumor production, on the kinetics and characteristics of dimethylnitrosamine demethylase and azo-reductase and the effect of mammary gland morphology on the types of tumors produced by different carcinogens.

Publications

Evarts, R. P., Brown, C. A. and Atta, G. J.: The effect of hydroxylamine on the morphology of the rat mammary gland and on the induction of mammary tumors by 7,12-dimethylbenz[a]anthracene. Exp. Molec. Pathol. 30: 337-348, 1979.

Evarts, R. P. and Brown, C. A.: 2,4-Diaminoanisole: Early effect on thyroid gland morphology and late effect on glandular tissue. J. Natl. Cancer Inst. (in press).

Mostafa, M. H. and Weisburger, E. K.: Effect of chlorpromazine hydrochloride on carcinogen metabolizing enzymes: Liver microsomal dimethylnitrosamine demethylase, 4-dimethylaminoazobenzene reductase and aryl hydrocarbon hydroxylase. J. Natl. Cancer Inst. 64: 925-929, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04619-15-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on the Metabolism of Chemical Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Preston H. Grantham	Chemist	LCM NCI
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	T. V. Reddy	Visiting Associate	LCM NCI
	R. Ramanathan	Visiting Associate	LCM NCI
	Peter P. Roller	Chemist	LCM NCI
	Elizabeth K. Weisburger	Chief	LCM NCI

COOPERATING UNITS (if any)
Analytical Chemistry Section, LCM, NCI; Nutrition and Metabolism Section, LCM, NCI

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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The metabolic pathways of chemical carcinogens, generally various aromatic amines, are studied both in animals or in isolated tissues. Metabolites are separated by various techniques such as thin-layer, column, gas-liquid or high pressure liquid chromatography. Identification is made through physico-chemical means such as mass spectrometry, nuclear magnetic resonance and ultraviolet spectra. Interaction of metabolites with cellular constituents is determined. The effect inhibitors or promoters of the carcinogens exhibit on the metabolic patterns is studied.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The aim of this research is to gather data relevant to the etiology of neoplasia at the molecular level. To this end chemical carcinogens, especially those of the aromatic amine type, are being utilized. Their metabolism and interaction with host tissues and specific targets are studied. Simplified yet realistic model systems are devised in order to gain an understanding of the fundamental events operating in chemical carcinogenesis. Specific inhibitors and accelerators of the carcinogenic process are applied as tools to develop information on and permit discrimination between biochemical events directly involved in the neoplastic change and those representing other reactions.

Methods Employed: Biochemical and pharmacological techniques are applied to determine the metabolism of 2,4-diaminoanisole, N-hydroxy-N-2-fluorenylacetamide, and related compounds in various animal species. This includes study of the enzyme systems concerned with certain metabolic steps. Procedures are developed and applied for the separation, purification and analysis of macromolecular constituents such as DNA, RNA, and proteins from tissues of animals treated with chemical carcinogens and from control animals. The metabolism of chemical carcinogens and related compounds, and interaction with host targets are examined in vivo and in vitro in the presence of various chemicals or antibiotics which affect the carcinogenic process.

Major Findings: (1) Study on metabolism of 2,4-diaminoanisole (2,4-DAA). Pretreatment with various enzyme inducers caused some quantitative changes in the excretion pattern of metabolites of 2,4-DAA. However, the extent of urinary excretion after a single dose of 2,4-DAA was similar in all groups pretreated with 3-methylcholanthrene (3-MC), α-naphthoflavone (BNF), 2,3,7,8-tetrachlorodibenzodioxin (TCDD) or phenobarbital (PB) in that approximately 66-75% of a dose was excreted in the urine within 24 hours. However, a more detailed time study showed that initially (5 hours) PB increased urinary excretion but by 24 hours the level in the untreated rats was almost the same. At the earlier time period PB increased significantly the water soluble metabolites, but at the 24 hour periods levels of all the metabolites were of the same order.

The metabolism of 2,4-DAA was followed in hamsters. At 24 hours after an i.p. dose of 2,4-DAA, the liver and kidney contained the largest amounts of radioactivity with lesser amounts in the spleen, lungs, heart, and testis. Workup in the usual fashion indicated that 2-acetamido-5-methoxy-1,4-benzoquinone was a urinary metabolite of 2,4-DAA in the hamster. To determine whether this was an artifact due to the isolation and workup procedure, the glucuronic acid fraction of the urine of hamsters which had been administered 2,4-DAA was worked up somewhat differently. Before enzymic release the fraction was acetylated and then hydrolyzed enzymatically. Workup by HPLC according to the usual procedures showed that the glucuronide fraction of urine of hamsters which had been acetylated contained a large amount (27%) as 5-hydroxy-2,4-diacetylaminoanisole. In nonacetylated urine this compound

accounted for only about 3% of the fraction. The methoxyacetamidoquinone accounted for about 15% of the fraction in urine worked up according to our usual procedures and none in the acetylated urine. These results indicated that the initial metabolite was probably 2-amino-4-acetamido-5-hydroxyanisole, present as a glucuronide. Probably during workup oxidation occurred with the subsequent isolation of the quinone. However, other experiments are being undertaken to check this point more thoroughly.

(2) Effect of phenobarbital. Urines from rats pretreated with phenobarbital before a dose of 2,4-DAA afforded a larger number of revertants in the Ames Salmonella system test for mutagens than did urines from groups treated with the other inducers such as 3-MC, BNF, or TCDD. The glucuronide fraction of these urines has been investigated using HPLC and TLC and the individual fractions were assayed for mutagens by the Ames test. On HPLC the mutagenicity seemed to disappear. Only one fraction contained a significant number of revertants, but it did not represent a significant amount of radioactivity or ultraviolet absorption. TLC separation of the metabolites in the glucuronide fraction showed the possibility of numerous more bands. However, none of the known metabolites of 2,4-DAA were represented by bands which had mutagenic activity. All the mutagenicity noted was concentrated in several bands which appeared to be fairly polar. Attempts to isolate and identify some of these mutagenic metabolites are underway.

(3) Mutagenicity of 2,4-DAA metabolites. The various metabolites of 2,4-DAA which had been identified were tested for mutagenic activity using the Ames system. 4-Acetylamino-2-aminoanisole, 2-acetylamino-4-aminoanisole, 4-diacetylacetylaminoanisole were all negative as mutagens. 4-Nitro-2-acetylaminoanisole and 2,4-dinitrophenol showed mutagenic activity.

(4) Water soluble metabolites of 2,4-DAA. In all cases "water soluble" metabolites of 2,4-DAA represent a significant portion of urinary metabolites. Attempts are being made to separate these materials by means of HPLC or TLC using radioactivity and mutagenicity assay in the Ames system to follow the metabolites. There were some quantitative changes following treatment with the various inducers, but qualitatively the same compounds appeared in the urines of rats regardless of the treatment with inducers. It is anticipated that this project to identify the water soluble materials will require a great deal of coordinated effort.

(5) Metabolism of 2-aminoanthraquinone (2-AAQ). During feeding of 2-AAQ drug metabolizing enzymes such as cytochrome P-450, epoxide hydratase and conjugating enzymes were increased. However, following the urinary and fecal metabolites of AAQ for several weeks of feeding, indicated that none of the major metabolites were mutagenic in the Ames Salmonella strains TA98 or TA100. Also 2-AAQ itself was not mutagenic. In all these studies the only fraction from AAQ feeding which had any mutagenic activity was found due to 1,2-diaminoanthraquinone, a minor contaminant of 2-AAQ which carried through the animals and was excreted as such. The diaminoanthraquinone was a potent mutagen; its mutagenicity was not influenced by glutathione or ascorbic acid. However, the presence of the 2 free amino groups was necessary to express mutagenicity.

The activation of 2-AAQ by BNF and PB induced microsomes and S-9 fraction from rats proceeded somewhat differently as measured by ethyl acetate extractable metabolites as separated by TLC. 2-AAQ, upon metabolic activation, led to metabolites which bound more to microsomal RNA than to microsomal proteins.

(6) Metabolism of N-2-fluorenylacetamide (FAA) in Fischer 344 rats injected with cytoxan. After injection of cytoxan the metabolism of FAA was altered somewhat. There was a reduction in the N-hydroxylation of FAA and its binding to microsomal macromolecules. The rate of binding was about 50% of the controls at one week after injection. Thus, although cytoxan along with FAA increases the incidence of bladder hyperplasia and possibly tumors, the percentage of activated metabolites and the binding to proteins, nucleic acids and the like was reduced. Sulfaguanidine also reduced N-hydroxylation of FAA and its binding to microsomal macromolecules. In general, sulfaguanidine depressed drug metabolizing enzymes.

(7) Hepatic vitamin A status of rats during AAQ feeding. It was found that feeding AAQ in the diet for different time periods increased the level significantly. The reasons for this decrease are not evident.

(8) Metabolism of 2,4-toluenediamine (2,4-TDA). The metabolism of 2,4-toluenediamine is being followed in rats which had been fed various enzyme inducers. Radioactivity in the various fractions and mutagenic activity are being used to follow the activated metabolites.

Significance to Biomedical Research and the Program of the Institute: By the utilization of certain chemical carcinogens the molecular mechanism of steps leading to cancer is being explored. If the entire process is viewed as a series of steps from the primary interaction between an agent and cellular constituents, followed by multiplication of abnormal cells which in turn can undergo further transformations, there are a number of points where one could prevent or even reverse such interactions. Thus, the ultimate aim of these studies is to comprehend fully the sequence of complex reactions leading to cancer and eventually to be in a position to prevent them under realistic conditions in man.

Proposed Course of Project: The program of elucidating the biochemistry of carcinogenesis, our eventual goal, is a continuing, long-term effort. Certain promising leads which have been discussed in this account will be followed up in further experiments.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04620-15-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mode of Action of Chemical Carcinogens - Chemical Investigations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Timothy Benjamin	Chemist	LCM NCI
OTHER:	Elizabeth K. Weisburger	Chief	LCM NCI

COOPERATING UNITS (if any)
Analytical Chemistry Section, LCM, NCI; Dr. Robert C. Atkins, James Madison University, Harrisonburg, VA

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.7	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project serves as a supporting function to the studies on metabolism of various toxic or carcinogenic agents in this Laboratory. The purity of compounds being investigated for toxicity or carcinogenicity is determined along with pertinent physical characteristics, useful in the design of the experiments. Possible metabolites of various aromatic amines, such as 2,4-toluenediamine, 2,4-diaminoanisole, and 2-aminoanthraquinone are synthesized as reference materials for metabolism studies. Methods for separation of aromatic amines and their metabolites are investigated.

Project Description

Objectives: In order to gain an understanding of the etiology of cancer, chemical studies are performed on the properties of certain carcinogens. Model studies on the reactions of chemical carcinogens with potential targets are performed.

Methods Employed: Properties of chemicals acquired or synthesized for the small scale bioassay of chemical carcinogens are investigated to determine the purity of these materials. In model experiments the reactions of certain of these agents, particularly their primary and ultimate reactive forms, with select potential targets are studied. The materials produced are made available for related studies on the biochemistry and biology of the carcinogenic process. Methods for purification and analysis of various chemicals are applied.

Major Findings: Supporting studies on synthetic reference materials for checking purity of compounds are continuing. Furthermore, by selective reduction of 2-methoxy-5-nitroacetanilide and separation by means of its copper chelate the N-hydroxy compound, 2-methoxy-5-(N-hydroxyacetamido)acetanilide, was obtained. The mass spectrum and the NMR spectrum confirmed this structure. With this reference material, which presumably is an activated metabolite of 2,4-diaminoanisole, it will be possible to identify additional metabolites of the parent compound. In addition, the question of whether the 5-hydroxy derivative of 2,4-diaminoanisole is formed by direct hydroxylation or by rearrangement of the N-hydroxy derivative can be undertaken.

Significance to Biomedical Research and the Program of the Institute: The studies performed are a necessary adjunct to the broad programs on the mechanism of action of chemical carcinogens pursued in this Laboratory.

Proposed Course of Project: This project will continue along the same lines as previously.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04680-10-LCM

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Development and Application of In Vitro Systems Involving Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. J. Wilson	Chemist	LCM NCI
OTHER:	J. Brown	Biological Lab. Tech.	LCM NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Carcinogen Metabolism, Carcinogenesis Intramural Program

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Epithelial-like cells derived from livers of infant Fischer rats are maintained in culture, either continuous or after frozen storage. Transformation of the cells to a malignant form by known chemical carcinogens and metabolites of known carcinogens is being studied.

Project Description

Objectives: Successful culture of epithelial-like cells derived from livers of 8 to 10 day old Fischer strain 344 rats and malignant transformation of such cultured cells by a variety of chemical carcinogens were discussed in previous reports. Since neoplasia in man and animals involves mainly epithelial cells, our principal aim in current investigations has been to further determine the potential of in vitro epithelial systems for use in rapid bioassay procedures for chemical carcinogens and in examining the biochemical mechanisms of carcinogenesis.

Two problems encountered in past investigations were the relatively long time required to develop cultures that were proliferating adequately to provide enough cells for biochemical investigations and the fact that some sublines of control cells underwent spontaneous malignant transformation as demonstrated by tumor formation following injection of cells into syngeneic hosts. Unfortunately, no distinctive morphological changes were apparent in the cultured cells that induced tumors. In addition, the spontaneous transformation was not correlated with a specific time in culture nor with the number of times a line had undergone subculturing. Therefore, we have emphasized the development of methods for producing a greater number of cultures with similar characteristics so that studies can be repeated as required in more replicable systems. Investigation of pertinent biochemical properties of untreated or chemically exposed cultured cells in respect to the effects of various cultural conditions and in comparison to the characteristics of liver tissue from whole animals should help assess the value of this in vitro system.

Methods Employed: Methods for selecting rat liver cells for culture continue to be developed in this laboratory. This involved detaching and transferring islands of epithelial cells, both mechanically and by trypsinization from cloning cylinders, so that many homogeneous sublines can be cultured from one primary culture. Previous studies in this laboratory have shown that frozen storage of cells did not alter enzyme levels or cause transformation.

Major Findings: (1) Treatment of rat liver cells with ethionine. Preliminary results indicate that irreversible morphological alterations can be produced in cultures of liver cells treated for three months with ethionine and that growth in soft agar is observed after five additional months in culture.

Significance to Biomedical Research and the Program of the Institute: An assay system which can be designed to give results more rapidly, specifically, and reliably than the customary long-term tests for carcinogenicity is highly desirable with the dual purpose of studying fundamental phenomena and the detection of harmful agents in our environment. Full exploitation of workable in vitro systems has not been achieved. It is our goal to develop such systems; an additional advantage of the in vitro systems is that eventually they can be established utilizing cells from man.

Hence carcinogenesis in vitro with human cells avoids the question of species difference, so often raised in evaluating the significance of carcinogen bioassay systems.

Proposed Course of Project: The epithelial cells will continue to be examined for suitability as a carcinogen screening system. Studies in progress with the carcinogen ethionine will be pursued. Collaborative work on the toxicity of chemotherapeutic platinum compounds will be initiated.

SUMMARY REPORT

LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY

October 1, 1979 through September 30, 1980

The major goals of the Laboratory of Cellular and Molecular Biology (LCMB) are to determine the etiology of naturally occurring cancers to elucidate mechanisms of transformation by carcinogenic agents and to develop experimental strategies capable of prevention of spontaneous and virus-induced tumors. The ultimate aim of these investigations is to apply approaches successful in animal model systems to the identification of causative agents of human malignancies and to the prevention of cancer in man. The primary emphasis of many ongoing investigations within the Laboratory concerns RNA tumor viruses. These viruses are unique among animal viruses in their mode of transmission and the intimate association that has evolved between these agents and cells of a large number and wide variety of vertebrate species. The etiologic role of this virus group has been established for naturally occurring cancers of many species, including some subhuman primates. Certain members of this virus group, so-called "replication-defective" transforming viruses, appear to have arisen by a mechanism involving recombination with cellular transforming genes. As such, these viruses offer an unparalleled opportunity to elucidate the processes by which such genes cause malignancies. Thus, research within the LCMB encompasses efforts to understand the processes involved in malignancy utilizing RNA tumor viruses as models. At the same time, studies are in progress to develop and apply the most sensitive and specific methods of tumor virus detection to the search for related oncogenic viruses of man. Major research efforts of the LCMB in these areas are centered within the Molecular Biology, Viral Genetics, and Animal Virology and Field Studies Sections.

During the past year, significant progress has been made by LCMB scientists in the investigation of replication-defective mammalian transforming retroviruses. These viruses have been isolated from a number of mammalian species, including subhuman primates. Our studies have led to the biological characterization of most of the known isolates of this virus group. In each case, these viruses have been shown to possess the capacity to transform cells, but to be replication-defective, requiring a type-C RNA virus as a helper. By a combination of approaches, scientists within the Molecular Biology Section have localized the region of one such virus, the Moloney murine sarcoma virus, responsible for transformation. This virus is known to be composed of some genetic sequence of its helper virus as well as some non-helper virus related genetic information. The latter shares sequence homology with the mouse cell genome. A physical map of the Moloney sarcoma virus was constructed by restriction enzyme analysis of the unintegrated linear viral DNA obtained from newly infected cells. Several restriction enzymes which cleaved linear MSV DNA 5' to the cellular insertion did not impair the transforming activity. In each case, the activity resided in the DNA fragments containing the intact cellular insertion. In contrast, digestion of the DNA with enzymes which cut within the cellular insertion

abolished the biological activity. By using molecularly cloned infectious viral DNA, we have extended these studies and found that certain enzymes, which cleaved 3' to the cellular insertion and within the terminal redundancy, did not impair transforming activity. These studies established that the cellular insertion in the Moloney-MSV genome is directly involved in the transforming activity of the virus.

Naturally occurring delection mutants have proven invaluable in the biochemical analysis of the genomes of viruses and cells. The knowledge that certain sequences of the MSV genome are dispensable with respect to its transforming function led us to inquire whether natural selection during virus replication and transmission might lead to the loss of nonessential viral information. Our approach consisted of cloning viruses after a single or multiple passage of Moloney-MSV-124 virus stock and characterizing their genomic size and expression of gene products. A series of nonproducer transformants isolated after infection at limiting dilution with the parental MSV-124 stock expressed at least two of the helper viral gag gene-coded proteins (p15 and p12), and in most cases, also p30. Analysis of the viral genomes in these transformants showed them to be of the same size of the parental virus (5800 nucleotides). In striking contrast, none of 20 transformants isolated after infection with an MSV-124 stock that has first undergone multiple cycles of virus infection expressed p30, and more than 80% lacked the ability to synthesize p12 as well. Indeed, the MSV genomes contained in these transformants were all found to suffer various deletions, involving up to 40% of the parental genome. Thus, during the course of multiple cycles of infection by MSV-124, the predominant population of biologically active virus has undergone a drastic selection towards a smaller genome size. Restriction enzyme analysis of the DNA of one of the mutants further narrowed the region of Moloney-MSV genome essential for transformation. We have now cloned several of these mutants in λ phage and are studying their biological and biochemical characteristics.

Detailed molecular analysis of the transforming gene requires the use of pure DNA in quantities not obtainable from *in vivo* synthesized MSV DNA. Scientists in the Molecular Biology Section have utilized recombinant DNA techniques to clone Moloney-MSV DNA. Unintegrated 5.8 kbp closed-circular DNA of Moloney-MSV-124 was isolated from acutely infected cells. This DNA was cleaved to genome-length linear form by the restriction endonucleases, Hind III or Xho I, and ligated to bacteriophage λ Charon 21A DNA, cleaved with the same enzyme. The recombinant DNA was packaged *in vitro* into phage particles and propaged in *E. coli* DP50SupF. Phage containing MSV-specific nucleotide sequences were identified by using molecular hybridization, and their DNAs were subjected to restriction enzyme and heteroduplex analyses. We obtained a total of five different MSV inserts of 6.4, 5.8, 5.2, 4.0 and 2.0 kbp, respectively. Whereas some of the plaque purified phages showed a single MSV insert, others contained several viral inserts of different size. We concluded that the propagation of MSV DNA in lambda phage generated a series of specific variants of the viral genome.

We have characterized these DNAs and found that the 6.4, 5.8 and 5.2 kbp inserts corresponded to MSV genomes with three, two and one terminal

repeats, respectively. The 4.0 and 2.0 kbp inserts were generated from the larger species by deletion of continuous segments of DNA starting in each case close to or at the end of the terminal repeat. The frequent generation of these deletions during growth in λ phage suggests that they are due to specific arrangement of nucleotide sequences within the viral genome.

Nucleotide sequence analysis is the most powerful tool to map structural genes and their control regions. We have undertaken the sequencing of the Moloney-MSV genome. We standardized three methods for DNA sequencing -- Sanger's dideoxy method, Maxam and Gilbert's chemical cleavage method, and Matt and Smith's nick translation method. These are being used in sequencing the MSV genome cloned in λ phage. More than 2000 nucleotides have been sequenced including the transforming gene in its entirety.

LCMB scientists have continued studies aimed at characterization of independent sarcoma virus isolates of the outbred cat. Studies by the Molecular Biology Section have shown that the Snyder-Theilen (ST), Gardner-Arnstein (GA) and McDonough (SM) strains of FeSV, like other mammalian transforming oncoviruses, were replication-defective, requiring a type-C RNA virus as a helper. Reproducible differences were demonstrated in the morphology of transformed foci induced by each FeSV strain.

The translational products of the three strains of FeSV have been comparatively characterized (a) by fractionation of cell extracts by gel filtration chromatography in the presence of 6M guanidine-HCl followed by analysis of appropriate fractions in competition radioimmunoassays for the main structural proteins of FeLV and (b) by immunoprecipitation of radiolabeled cell extracts with antibodies directed against FeSV-coded specific polypeptides. The results demonstrated that the main FeSV gene product is a polyprotein of both type-C helper viral and cellular origin. Each polyprotein contains the amino terminal moiety of the helper FeLV gag gene (p15, p12 and a portion of p30 in ST- and Ga-FeSV, and p15, p12 and p30 in SM-FeSV) fused to a sarcoma virus-specific polypeptide. With at least one FeSV strain (ST-FeSV), we have shown that the sarcoma virus-specific domain of the polypeptide appears to be derived from a fragment of a normal cellular protein so far found to be present in all Felidae species as well as in some other carnivore genera. These findings support the concept that the host sequences present in the ST-FeSV genome are part of an active cellular gene rather than a non-informational segment of the feline cellular genome.

The relationships between the three strains of FeSV were also investigated. Molecular hybridization experiments revealed that the sarcoma virus-specific sequences of ST- and GA-FeSV, but not those of SM-FeSV, are related. Similarly, antibodies elicited against the sarcoma virus-specific domain of the ST-FeSV coded polyprotein recognize the corresponding region of the GA-FeSV, but not of the SM-FeSV, translational product. These findings indicate that in naturally occurring tumors of outbred species, as previously found with those of laboratory animals, different cellular genes can be involved in the generation of replication-defective transforming viruses.

We have developed cDNA probes specific for the FeLV-A, -B and -C subgroups as well as radioimmunoassays capable of detecting type-specific antigenic determinants in their respective gag gene-coded p15 proteins. These techniques have allowed us to determine that different FeLVs were involved in the independent generation of ST-, GA- and SM-FeSV strains. Our results indicated that FeLV subgroup B was involved in the generation of ST-FeSV. However, neither of the known subgroups appeared to be responsible for the generation of GA- and SM-FeSV. Whether this is due to the presence in nature of other subgroup variants of FeLV or to genetic changes that have occurred in these sarcoma viruses after their generation remains to be explored.

Research efforts were directed toward establishing evidence for the involvement of the FeSV-coded polyproteins in cell transformation. To date, genetic experiments have consistently shown expression of the ST-FeSV coded polyprotein (ST Pp85) in ST-FeSV transformants but not in revertant subclones. More suggestive evidence for a role of ST Pp85 in cell transformation comes from our recent findings demonstrating the presence of a protein-kinase activity associated with ST Pp85. This protein kinase has properties similar to that of RSV-coded pp60^{src}. The enzyme possesses in vitro phosphorylating activity for immune IgG specifically directed against certain domains of the ST Pp85 molecule, as well as for ST Pp85 itself. Experiments designed to further characterize this enzymatic activity as well as to definitively demonstrate its intrinsic association with ST Pp85 are underway. More significant information should be obtained from efforts aimed at the isolation of this sarcoma virus-coded protein and the investigation of its putative oncogenic effect upon insertion (by means of liposomes, microinjection, etc.) into suitable cells.

Oncoviruses have been shown to be etiologically involved in naturally occurring tumors of a wide variety of vertebrate species. They appear to exert their oncogenic potential under conditions in which there is poor host cell control of virus replication. This is the case for horizontally transmitted oncoviruses of birds, rodents, arlyodictyles, carnivores and primates.

It has become evident that oncoviruses can interact with their hosts in a manner which appears to be unique among viruses of vertebrates. In many species, including primates, oncoviruses are transmitted from one generation to the next, often in an unexpressed form, within the host cell genome. Under such conditions, these endogenous viruses appear to be subject to regulatory processes analogous to those affecting cellular genes. Over the past several years, our laboratory has isolated a number of new endogenous oncoviruses. Moreover, we have investigated the relationships of such viruses to known oncoviruses as well as the distribution of related viral sequences within vertebrate cellular DNAs by molecular hybridization techniques.

We have applied radioimmunological techniques to demonstrate evolutionary linkage among distantly related oncoviruses by radioimmunological techniques. Antibodies elicited against proteins of a given virus often bind analogous proteins of oncoviruses isolated from species belonging to different Orders

or Families of mammals. The designation "interspecies" has been applied to antigenic determinants recognized by an antibody in this kind of reaction. Broadly reactive interspecies antigenic determinants have been demonstrated in the major structural proteins of all known mammalian type C viruses. These findings have been extended to include other translational products representing more than two-thirds of the type-C viral coding capacity. All of these studies have led to the conclusion that mammalian type-C viruses arose from a common progenitor.

During the past year, studies within the Molecular Biology Section have shown that the guinea pig retrovirus (GPV) is an endogenous virus of both domestic and indigenous guinea pigs. No relationship could be found between the GPV genome of the cellular DNAs of other hystricomorphs, in particular, and other rodents or mammals in general. The results suggest that guinea pigs have acquired this endogenous oncovirus as a result of a relatively recent interspecies transfer.

The GPV major internal protein, p26, was purified by sequential phosphocellulose and gel filtration chromatography. Interspecies reaction observed when ^{125}I-labeled GPV p26 was incubated in the presence of antisera elicited against a variety of oncoviruses. They included the binding of GPV p26 by anti-VRV and anti-squirrel monkey virus (SMRV) sera. In reciprocal experiments in which anti-GPV serum was tested for its ability to immunoprecipitate a variety of ^{125}I-labeled oncoviral proteins, antigenic cross-reactivity between GPV p26 and the major internal protein of mouse mammary tumor virus, MMTV p26, was found. Thus, GPV shares interspecies antigenic determinants with analogous proteins of representative members of both type-D (SMRV) and type-B (MMTV) oncoviruses, as well as with VRV.

Avian reticuloendotheliosis viruses (REV) have been shown to possess ultrastructural features that resemble those of mammalian type-C viruses. Moreover, viruses of the REV group possess an RNA-dependent DNA-polymerase, whose molecular weight and cation preference are similar to those of murine type-C viruses. Finally, the REV major internal antigen, p30, has been shown to exhibit amino acid sequence homologies with the corresponding proteins of Rauscher-MuLV and FeLV. We demonstrated that REV-A p30 as well as the corresponding protein of other members of the avian REV group possess a subset of interspecies antigenic determinants shared by all known mammalian type-C viruses. Direct immunocrossreactivity between REV-A p30 and the p30s of several mammalian type-C viruses was also demonstrated. These findings provide strong evidence for the mammalian origin of the nongenetically transmitted avian reticuloendotheliosis virus group.

We have also investigated the immunologic relationships between OMC-1, an endogenous oncovirus of owl monkeys, with representatives of the three oncoviral genera by radioimmunologic techniques. The major structural protein of OMC-1 was shown to share antigenic determinants with the corresponding proteins of certain type-C viruses of rodent, feline and cervine origin. It was not possible to demonstrate antigenic cross-reactivity between OMC-1 and other endogenous primate type-C or type-D isolates, arguing against the concept of a single common ancestor virus for the known

endogenous oncoviruses of primates. A close antigenic relationship, however, was established between OMC-1 and avian reticuloendotheliosis viruses. These results suggest that OMC-1 may be in the evolutionary lineage that led to the generation of viruses capable of crossing the inter-Class barrier between mammals and birds.

We sought to elicit evidence of immunological links among type-B, type-C and type-D viruses, representing the three known genera of the Oncovirinae subfamily of retroviruses. The major structural proteins of a large variety of viruses were purified by a series of chromatographic techniques carefully selected with emphasis on preserving, as much as possible, the antigenic determinants of each molecule. Goats and rabbits were immunized with isopycnically purified oncoviruses. Biweekly bleeds were systematically tested for the presence of antibodies directed against the generally weak immunogenic interspecies antigenic determinants. We found that in some cases, such antibodies were only transiently detected. In others they were present, in low titers, for most of the eight-month immunization period.

This systematic approach allowed us to detect two subsets of cross-reactive antigenic determinants among the three oncoviral genera. One subset is shared by the major structural proteins of type-D viruses and mouse mammary tumor virus, the prototype type-B virus. The second subset is also present in the major structural proteins of the type-D virus group, and was shared with analogous proteins of certain mammalian type-C viruses, particularly those of rodent origin. To date, there is not sufficient evidence to determine whether all members of the Oncovirinae subfamily of retroviruses have diverged from a common progenitor or have arisen by recombination of ancestor viruses of independent evolutionary lineage. Whichever mechanism has been more important, our findings establish strong evidence for evolutionary relatedness among the three well-defined oncovirus genera.

As we have developed new biological, biochemical and immunologic techniques for detection of oncovirus expression, these techniques have been applied to the search for oncovirus expression in man. For example, we have developed very broadly reactive competition radioimmunoassays that detect most, if not all, known oncoviruses. These assays might be expected to be useful in the detection of antigenic determinants of new oncoviruses. Similarly, the analysis of new virus isolates has been aided by the development of highly type-specific radioimmunoassays that can discriminate between very closely related virus strains. Immunoassays have also been applied to epidemiologic studies, in which we have searched for antibodies to oncoviruses in man.

Recently, Kurth and Mishky (PNAS 75: 5692-5696, 1978) reported that a large fraction of human sera were capable of precipitating at relatively high titers the purified major envelope glycoprotein (gp70) of the woolly monkey type-C virus. In collaboration with D. Bolognesi, Duke University, we were able to demonstrate that using the experimental assay conditions utilized by these authors, antibodies in human sera capable of precipitating a wide variety of oncoviral glycoproteins could be detected. However, substances as diverse as normal components of serum, extracts of bacteria and even non-protein molecules such as glycogen also shared the oncoviral

glycoprotein determinants recognized by normal human sera. It was found that immunoprecipitation of a given viral glycoprotein by human sera was entirely dependent on the cell in which the virus was grown. Human sera specifically did not recognize glycoproteins purified from oncoviruses grown in human or higher primate cells. These findings not only demonstrate that the antibodies were directed against cellular rather than virus-coded antigenic determinants, but also exclude the possibility that this immune response was elicited as a consequence of oncovirus exposure. Our studies as well as complementary findings of Snyder and Fleissner (PNAS, in press) raise a note of caution that should be taken into account in future efforts aimed at detection of antibodies to oncoviruses in man.

Research within the Molecular Biology Section is also aimed at elucidating how leukemia viruses cause malignancies. Mouse leukemia viruses have been reported to induce tumors involving cells within the T lymphocyte lineage. We found striking differences in the target cells for in vivo transformation by two clonal replication-competent type-C viruses, Moloney- and Rauscher-MuLV. Moloney-MuLV induced tumors and lymphoma cell lines exhibited Thy-1 antigen in the absence of detectable Fc or C3 receptors, indicating their T cell origin. However, Rauscher-MuLV primary tumors and lymphoma cell lines of the same mouse strain invariably exhibited Fc receptors in the absence of Thy-1 antigen, suggesting that these tumors were of the B lymphoid cell lineage. The pattern of immunoglobulin synthesis by individual Rauscher-MuLV tumor cell lines was determined both by biosynthetic and radioimmunologic techniques. Rauscher-MuLV lymphoma lines invariably expressed immunoglobulin heavy (μ) chain in the absence of detectable light (μ or κ) chains. All of these findings established that the target of neoplastic transformation in response to Rauscher-MuLV is an immature cell within the B lymphoid lineage.

These findings have direct bearing upon possible mechanisms of transformation by this virus group. Current models favor either a mechanism that involves transformation of the infected target cell or one in which transformation is mediated through some other virus action on the host. Possible indirect mechanisms include blastogenesis of lymphoid cells in response to virus gene products, leading to the eventual selection of a malignant clone. Our findings argue that blastogenesis in response to different leukemia viruses would have to be very specific in order for this mechanism to explain the reproducibly distinct lymphoid target cells for transformation by the different leukemia viruses.

An important genetic approach for analysis of the viral gene(s) involved in leukemogenesis might result from the generation of recombinants between an oncogenic mouse type-C virus and an endogenous virus without demonstrable malignant potential. Using a double selection system devised to select against the temperature-sensitive (ts) lesion associated with a mutant of Rauscher-MuLV and a xenotropic host range of an endogenous mouse type-C virus (BALB:virus-2), recombinant viruses were obtained at frequencies ranging from 0.01 to 0.1%. Recombinant viruses were identified on the basis of the type-specific antigenic determinants in the translational products of gag, pol and env genes. By this approach, the partial genetic maps of a

large number of recombinants were obtained. The fact that p10 of Rauscher-MuLV ts 25, the mutant utilized, was the only protein uniformly lacking in recombinant viruses localized the lesion inhibiting gag precursor cleavage in this mutant at the carboxy terminal.

The recombinant viruses demonstrated two host range phenotypes as defined by Fv-1 host cell restriction. In each case, NB-tropic recombinants possessed the p30 of the Rauscher-MuLV parent, whereas the rest, N-tropic and host range, exhibited BALB:virus-2 p30. Thus, it was possible to assign the site of Fv-1 action at, or closely linked, to the viral p30. The target within the viral genome of a second host restriction was also mapped. A serum factor previously shown to specifically inactivate xenotropic virus infectivity was demonstrated to exert infection on the viral env gene product. The recombinant viruses generated are currently being tested to determine what region of the viral genome are essential for oncogenicity. Efforts are also under way to generate recombinant viruses between parental viruses that transform T and B cells, respectively. It is hoped that this approach will be useful in mapping the region of the viral genome responsible for transformation of specific target cells.

Research within the Viral Genetics Section has been aimed at characterizing gene products of FeSV strains as well as of the Abelson leukemia virus (AbLV), a replication defective lymphosarcoma virus of mouse origin. Studies were performed in an effort to exclude the possibility that synthesis of AbLV P120 might have resulted from a gag/pol deletion. Such a deletion could encompass sequences encoding most of p30, p10 and sufficiently extensive portions of the viral reverse transcriptase to preclude its detection by competition immunoassay. Three methionine-containing tryptic peptides present in Moloney MuLV Pr65gag were each shown to be present in both Pr75gag and in Pr180gag/pol. Of these, one peptide, corresponding to Moloney MuLV p12, but neither of two p30 specific peptides were present in AbLV P120. The twelve remaining methionine-containing tryptic peptides were found to be unique to AbLV P120 and not shared by either Moloney MuLV Pr180gag/pol or Pr82env. These findings thus strongly argue that the AbLV genome was derived by genetic recombination between Moloney MuLV genomic RNA and host cell specific transforming sequences and that the latter sequences encode the nonstructural components of AbLV P120.

Tryptic peptide analysis of polyproteins encoded by the three independently derived FeSV isolates led to the demonstration of five methionine-containing peptides specific to the nonstructural portion of Gardner FeSV P115, three of which are also represented in Snyder-Theilen FeSV P115. None of these [^{35}S]-methionine labeled tryptic peptides were present in translational products representative of the complete FeLV subgroup A genome, including Pr180gag/pol, Pr65gag and Pr82env. These findings provide further evidence that the non-structural components of such polyproteins are encoded by acquired cellular sequences.

A genetic approach has been pursued to further characterize the above described virus coded polyproteins and determine the significance of their associated protein kinase activities and their role in transformation. Both

AbLV and Gardner FeSV transformed mink cells were found to be subject to a high frequency of morphologic reversion to a nontransformed phenotype. Such spontaneous revertant clones are defective in cellular functions influencing expression of transformation. Loss of expression of the transformed phenotype was accompanied, in each case, by a reduction of polyprotein expression to nondetectable levels arguing for a requirement of the polyprotein for expression of the transformed phenotype. In addition to the spontaneous revertant clones, a large number of transformation-defective (td) AbLV mutants have been obtained. Cells nonproductively infected with such mutants exhibit a high degree of growth contact inhibition, fail to form colonies in soft agar, lack rescuable transforming virus and are equally as susceptible as uninfected control cells to transformation by wild type AbLV pseudotype virus. In addition, each of several td AbLV nonproductively-infected cell clones tested was found to be nontumorigenic in a syngeneic host. Biochemical analysis of such clones revealed expression of the major AbLV translational product, P120, at concentrations comparable to those in wt AbLV transformed cells arguing that, although AbLV P120 expression may be necessary for expression of transformation, it is not sufficient. A protein kinase enzymatic activity associated with AbLV P120 in wt AbLV transformed clones was diminished to below detectable levels in the td AbLV mutant nonproductively-infected clones. These findings indicate that the AbLV P120 and its associated protein kinase activity are involved in AbLV tumorigenesis.

In other studies, scientists in the Viral Genetics Section have utilized nonstructural replication mutants of SMRV in combination with sensitive radioimmunologic assays, to identify a 72,000-molecular-weight precursor polyprotein (Pr72^{gag}). Post-translational cleavage of Pr72^{gag} gives rise to virion structural proteins of 35,000 (p35), 16,000 (p16), 12,000 (p12) and 9,000 (p9) molecular weight. Three of these viral proteins, p35, p16 and p9 have been shown to be phosphorylated. Analysis of viral antigen expression in cell lines nonproductively infected with either of two replication-defective SMRV mutants or mink cells productively infected with wild type SMRV resulted in the detection of several SMRV Pr72^{gag} intermediate cleavage products. Adjacent proteins within such cleavage products were identified and the intracistronic order of individual structural proteins within SMRV Pr72^{gag} was tentatively deduced as NH_2-p16-p12-p35-p9-COOH.

LCMB efforts to elucidate mechanisms of carcinogenesis are complemented by the In Vitro Carcinogenesis Section. Research of this Section is aimed at determining mechanisms of spontaneous and carcinogen-induced malignant transformation of cultured cells of rodent and human origin. Special emphasis is being directed at the development of culture systems utilizing human epithelial cells to study the interactions of chemical carcinogens and viruses with cellular DNA. Further efforts are aimed at defining the fundamental cytologic, biologic, and biochemical characteristics of carcinogenic change.

The environment in which cells proliferate, function, and/or transform is continuously altered by the cell population. Earlier studies in this Section linked two ubiquitous components of the environment, oxygen and

visible light, with changes in chromosome structure and number. Concurrent studies implicated the type of serum used to supplement the growth medium with spontaneous neoplastic transformation of rodent cells (i.e., transformations that occur regularly without addition of a known carcinogen or virus). The following studies were designed to quantify the biologic effect(s) of oxygen, visible light, and serum type, in order to understand mechanisms of cell injury, response and transformation. Murine systems were used because: (a) of the vast body of genetic information available on inbred strains of mice; (b) means of assaying tumorigenicity of cultured cells in syngeneic hosts; and (c) the frequency with which chromosomal alterations and transformation are observed in mouse cells. Human systems were selected because of our interest in using human cells for in vitro carcinogenesis and mutagenesis assays; where this proved technically difficult, monkey cells have been used.

Susceptibility of mouse cells to light-induced chromatid damage was shown to increase with prolonged culture and is associated with transformation. This increased susceptibility could conceivably result from 1) loss or inactivation of defense enzymes such as catalase or superoxide dismutase or 2) impaired DNA repair. No consistent change in catalase activity with respect to time in culture, neoplastic state or growth phase could be established. To explore the second possibility, cytogenetic damage was interpreted in terms of DNA strand breaks. Whereas both nonneoplastic and transformed cells are susceptible to DNA damage during G_1, only transformed cells are susceptible during late S-G_2. Studies with caffeine show that the increased susceptibility of transformed cells results from impaired capacity to repair DNA damage.

Scientists with the In Vitro Carcinogenesis Section devoted considerable effort to defining growth requirements of normal human epithelial cells and developing methods for producing adequate numbers of cultures for carcinogenesis studies. A new medium, NCTC 168, designed for human skin epithelial cells was formulated. When used with horse serum, NCTC 168 supports initiation and continued epithelial cell outgrowth in the absence of irradiated rodent or human feeder cells. This medium also supports passage to the 6th subculture without feeder cells, and EM studies show the presence of desmosomes and tonofibrils characteristic of keratinocytes.

Exposure of human fibroblasts to fluorescent light has two diverse effects. Short intermittent exposure reproducibly enhances proliferation rate and significantly extends lifespan. Stimulation is mediated through the culture medium and more specifically by light-exposed amino acids tyrosine and phenylalanine. The effective wavelength lies between 290 and 500 nm. The second influence of light is on chromosomal and DNA damage. Like nonneoplastic mouse fibroblasts, normal human cells are susceptible to light-induced chromatid damage; however, susceptibility does not increase with time in culture. Transformed human fibroblasts and skin fibroblasts from patients with Gardner's syndrome, xeroderma pigmentosum and ataxia telangiectasia are markedly more susceptible or develop increased susceptibility during culture. The damage in human fibroblasts results from excess H_2O_2 generated in the cell, is almost completely prevented by catalase

and is significantly decreased by addition of selenite or mannitol during light exposure. Selenite may be necessary to maintain sufficient activity of the selenoenzyme, glutathione peroxidase, and mannitol scavenges free hydroxyl radical, $\cdot OH$. Thus $\cdot OH$ and H_2O_2 formed within the cell during light exposure are agents that directly or indirectly cause the chromatid damage.

Low intensities of fluorescent light were shown to cause not only chromatid breaks but also an estimated 150 DNA crosslinks per cell under normal growth conditions. These crosslinks are between DNA and protein, and their formation is affected by a variety of conditions. Oxygen enhances crosslinking and glutathione with ascorbic acid inhibits it; increased light intensity increases crosslinks; cross-linking is medium-mediated; the extent of crosslinking is influenced by cell density in that high cell densities decrease crosslinks; addition of catalase during illumination does not influence crosslinking; light of 365, 405 or 436 nm wavelength separately is not responsible for the crosslinking, and the effect of cutoff filters suggests an effective wavelength between 450 and 490 nm. Since proteinase K added to cell extracts eliminates crosslinks, DNA and protein linkage is implicated. The DNA-protein crosslinks are repaired in about 24 hrs, and repair is inhibited by caffeine, suggesting a postreplication repair mechanism.

Intermittent exposure to low-intensity fluorescent light protects human skin epithelium in culture from the cytotoxicity of benzo(α)pyrene (BP). Light exposure also reduced dramatically the covalent binding of BP to cellular DNA, inactivated 3-hydroxyl BP hydroxylase, and destroys BP in the medium. Even though BP is destroyed by light, light also produces covalent binding of BP products to purified DNA under culture conditions. Low levels of BP (0.1 μg/ml) bound to cellular DNA independently of light exposure and formed stable adducts not removed during a subsequent week of incubation. Conceivably, DNA damage from chronic low level exposure to BP in the environment could accumulate in human epithelial cells to unexpectedly high levels.

Investigations in the laboratory over the past several years have suggested that prevention of spontaneously and chemically induced cancers in animal model systems might be accomplished by active or passive immunization with appropriate preparations specific for tumor cell antigens held in common by various neoplasms of a given species. Efforts have focused on the determination of which endogenous viral gene products might be the most effective immunogens, utilizing tumor prevention as the end point. Achievement of this goal not only fulfills the pragmatic objectives of cancer prevention but at the same time may provide important insights into mechanisms involved in the causation of cancer. These efforts have broadened in scope to include the search for shared tumor antigens that may be coded by endogenous transforming (src) genes. The Viral Immunology Section has the major responsibility within the LCMB for research in these areas.

Spontaneous lymphoma was prevented in the mouse by either active or passive immunization. The mechanism of this protection appears to be by prevention

of expression of the endogenous ecotropic virus. Protection was not obtained when IgG was used which had a high neutralizing titer against the murine xenotropic virus. Future studies in this area will probably be restricted to defining the role of the ecotropic virus in initiating the leukemogenic process.

Chemically-induced fibrosarcomas were prevented in the mouse by either active or passive immunization. The successful vaccine was radiation leukemia virus (a recombinant virus) or antibody prepared against it. The antibody studies demonstrated that high titered antibody against only ecotropic or xenotropic viruses or admixtures of these two did not protect. The only antibody which did work had both anti-ecotropic and xenotropic activity simultaneously. However, the above studies argue that the active factor was probably antibody against something else. The current working hypothesis is that the RadLV is also expressing the "oncogene" product and that host response to this antigen provides the protection observed. Current and future studies will focus on identifying what is the active component in the RadLV that induces this protection.

In a second closely related area, various cell vaccines are being created and evaluated for their ability to successfully immunoprevent chemically-induced tumors. The AKT-8 cell appears to protect. The success of this system and others would demonstrate the generality of introducing the species-specific oncogene into a heterologous cell, by means of a viral vector, where its expression in that cell would lead to immunization and prevention of neoplasia.

Almost 30 different human tumor cell lines were injected into ATS-immunosuppressed newborn rats. Many of the cell lines resulted in growth of a tumor. These tumors were serially transplanted. Complement fixation testing, karyology (kindly performed by Dr. Walter Nelson-Rees, University of California, School of Public health, Oakland, California), analysis of tumor formation and assay for expression of the rat "src" sequence (kindly performed by Dr. Howard Young, NCI-FCRC) showed that after passage approximately 5-10 times the tumors all converted from human to rat. Repeated injection of normal human cells into ATS-treated rats or of human tumor cells into non-ATS-treated rats did not result in the formation of any tumors in the rat.

There are indications that in certain types of human neoplasia (especially familial polyposis) an inheritable, dominant gene is involved in determining susceptibility to development of neoplasia. The segregation of this gene can be followed within families. Expression of this gene also correlates with ease of transformation of these cells by a virus. Development and verification of a transformation assay may lead to a rapid and easy method for screening whole human populations to define those people at high risk for developing cancer. Efforts have been made to establish cell lines from human donors and then determine the ease with which these cells are transformed by various candidate viruses. These results can then be correlated with clinical observations of the donor by a physician. Another facet of these _in vitro_ studies is the viral infection of known human tumor

cells to activate and/or rescue the human src sequence in such a form that it can be used as a vaccine or transferred into a heterologous cell which can be used as a vaccine.

Development of reproducible testing systems for identification of relative genetic susceptibilities to cancer is much needed in cancer diagnosis. Assuming that phenotypic expressions that appear in cell strains reflect its biological abnormalities, the study of cultured skin fibroblasts derived from individuals with an inherited form of cancer such as adenomatosis of the colon and rectum (ACR) provides a unique system for analysis of the oncogenic process. The occurrence of tumorigenesis-related phenotypic markers has recently been demonstrated in these skin fibroblasts. Other markers identifying high autosomal dominant risk include polyps and adenomas of the colon as well as subcutaneous cysts.

High-titered ($>10^5$) Ki-MSV stock viruses were prepared for use as viral assays of relative risks (+) or (-) in cancer family syndromes such as ACR and Gardner's. Freshly harvested supernatant fluids of Ki-MSV transformed NRK cells were subjected to a density gradient purification using zonal ultracentifuges with RNAs-free sucrose gradients, and the banded, purified virus was resuspended in Tri buffer (pH 7.0) at concentration of 500 X.

Human skin cell lines (Risk + and Risk -) furnished by Drs. Rasheed and Kopelovich were tested for focus formation by Ki-MSV. The results confirmed the genetic differential susceptibility of cultured human skin cells to transformation by Ki-MSV. Thus our tests identifying quantitative high risks in autosomal dominant ACR and Gardner syndromes from Kopelovich and Rasheed provided confirmation of high and low cancer risks in three laboratory test systems, thus providing the basis for clinical management of subsequent colon cancers.

In the past year the Bio-molecular Oncology Section became part of the LCMB. The primary goal of the research of this Section is to study physiological induction of endogenous retroviruses and their subsequent potential influence on normal host functions. One way by which retroviruses might influence host function is by the induction of a cell regulatory protein(s). One of the best characterized class of virus induced cell regulatory proteins is the interferons. In addition to their potent antiviral activity, this class of glycoproteins is rapidly becoming recognized for their diverse biological affects. Although retroviruses are not considered to be potent interferon inducers, we examined extracts prepared from reproductive tissues at various times during gestation for antiviral activity by the reduction of encephalomyocarditis virus (strain MM) induced plaque formation in L929 cells. Our initial studies revealed the presence of significant antiviral activity (PR_{50}=2500 units/ml) in mouse term placental extracts. This activity was sensitive to treatment with trypsin, actinomycin D and heat, and was neutralized by anti-interferon gamma globulin. Also, it was not inactivated by low pH treatment (pH 2) and was not removed by high speed centrifugation. By these criteria, the placental component

exhibits the properties of viral induced (Type 1) interferon. During gestation, interferon was detected in most uterine specimens between 10-15 days after mating; however, samples taken before or after this interval of pregnancy, as well as from virgin mice, had no detectable titer (<10 units/ml). The transient appearance of interferon in the gravid uterus developed abruptly and was highest at the 13-day stage (300 units/ml). In comparison, interferon was found in all placental samples and its level increased significantly during gestation (>2500 units/ml). Embryonic and fetal specimens had little detectable antiviral activity.

It has been suggested that prolonged retrovirus gp71 stimulation of lymphocytes, on the one hand, combined with viral-induced immunodepression, on the other, could account for the initiation and continued progression of neoplastic disease in the lymphoid system. Model studies have been initiated by scientists of the Bio-molecular Oncology Section to examine the interaction of R-MuLV-gp71 with its receptor on immunocompetent lymphoid cells, to characterize the receptor and to evaluate the significance of this interaction for normal and immunopathological phenomena. The major envelope glycoprotein (gp71) of R-MuLV binds rapidly and with high specificity to murine lymphoid cells. Binding is inhibited by antiserum to R-MuLV gp71 and is competed by either murine ecotropic virus (R-MuLV and Friend, or F-MuLV) or the viral envelope glycoprotein (gp71) purified from R-MuLV. In contrast, murine xenotropic viruses (NZB and M55) fail to interfere with R-MuLV gp71 adsorption to lymphoid cells, indicating that mouse lymphoid cells, like mouse fibroblastic cells, contain a population of membrane receptors for ecotropic virus that have no demonstrable affinity for mouse xenotropic virus. The availability of membrane receptors for R-MuLV gp71 on lymphoid cells is also reduced by exogenous infection of susceptible mice with either R-MuLV or F-MuLV. The reduction of binding occurs shortly after infection and the progressive loss of available receptors is highly dependent on the replication of virus in the challenged host.

The active binding region of the gp71 molecule is associated with a fragment of less than 30,000 daltons (SDS-PAGE analysis). This fragment contains nine of the fifteen major tryptic peptides of gp71 and all three glycopeptides associated with gp71 appear to be contained within this component. The fragment exhibits cellular binding kinetics similar to the native glycoprotein except that the divalent cation requirements for optimum binding are less stringent. For gp71 cellular binding there is a high requirement for Ca^{2+} which in part, is replaced by either Mn^{2+} or Cd^{2+} but not by Mg^{2+} or Zn^{2+}. By comparison, substantial binding of the small molecular weight component to cells occurs in the absence of divalent cations and with their addition, binding capacity is only moderately elevated. The binding of the active component to cells is competitively inhibited with unlabeled gp71 and is prevented by specific antiserum to R-MuLV gp71. Furthermore, the kinetics of inhibition for both competition reactions appear identical to that obtained using purified R-MuLV gp71.

Scatchard plot analysis of gp71 binding to normal murine thymocytes indicates the existence of a single affinity class of receptors for ecotropic

viruses which exhibit no cooperative interactions. The estimated equilibrium association constant (Ka) of this population is approximately $2x10^{10}M^{-1}$, indicating that the binding affinity of gp71 by murine thymocytes is greater than cultured murine fibroblastic cells ($8.3x10^{9}M^{-1}$). By comparison the number of gp71 molecules bound per murine lymphoid cell ($0.8\text{-}2.4x10^{4}$) is substantially less than cultured fibroblast ($4\text{-}5x10^{5}$) and varies with mouse strain, cell type, age, leukemogenesis and pregnancy.

In addition to their intramural research efforts, investigators within the LCMB serve on the editorial boards of major journals in their field, serve as members of various grant review bodies, and participate in a large number of collaborative efforts with scientists in laboratories throughout the country. The major goal of studies within the Laboratory is to apply, wherever possible, basic information derived from a multi-disciplinary approach to the study of virus-induced and spontaneously occuring cancers to its most important application, the prevention of cancer in man.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04805-10 LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cocarcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Hellman	Assistant Chief	LCMB NCI
Other:	A. K. Fowler	Head, Bio-molecular Oncology Section	LCMB NCI
	D. R. Twardzik	Biochemist	LCMB NCI
	P. T. Allen	Microbiologist	LCMB NCI

COOPERATING UNITS (if any)
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Bureau of Radiological Health
Rockville, MD 20857

Litton Bionetics, Inc.
Frederick Cancer Research Center
Frederick, MD 21701

LAB/BRANCH: Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: DCCP, NCI, NIH
Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are designed to provide information on which to estimate the contribution of physiological stress to the process of transformation and malignancy. Model systems consisting of rodents, as well as cell cultures are being used to evaluate the modification of normal host physiology on subsequent cancer occurrence. In particular, tumor promoters, exposure to X-ray and ultraviolet light are being investigated.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

Tumor promoters are a group of compounds that by themselves do not induce cancers, but markedly enhance the production of malignancies if administered at times after exposure to low doses of carcinogen. The mechanism of initiation and promotion is not well understood. We are attempting to elucidate mechanisms of tumor promotion utilizing virological and immunological techniques.

Methods Employed:

Cell exposure to tumor promoters, U.V. and X-irradiation and evaluation of the subsequent response is being studied in vitro. Animal exposure to promoters is being studied by evaluating tumor induction, as well as cellular and humoral immune capacity.

Major Findings:

In collaborative studies with investigators of the Bureau of Radiological Health (FDA) we have observed the activation of endogenous retroviruses in murine $S^{+}L^{-}$ cells by tetradecanoyl phorbol acetate (TPA), a protease inducer. It has been proposed that proteases produced in response to promoting agents activate genes by altering regulatory proteins that block their expression. Supporting this concept, we have demonstrated that protease inhibitors (antipain and leupeptin) block retroviral induction by TPA, IDUR and U.V. inactivated Herpes virus. More recently, we have also demonstrated the independent mitogenic activity of TPA for murine and nonhuman primate lymphocytes and have noted that the mitogenic response to TPA in mice is strain and dose dependent. Moreover, we observed TPA-induced potentiation of mixed lymphocyte reactivity and polyclonal mitogen stimulation. TPA is now being employed in long-term cultures of baboon T-lymphocytes in an effort to enhance the activation of endogenous retrovirus and to evaluate the influence of this cocarcinogen on T and B-lymphocyte interaction. In related studies, we have found TPA-induced production of an immunodepressive factor(s) by murine macrophages.

Significance to Biomedical Research and the Program of the Institute:

The protease inhibitor data suggests that protein suppressors of virus expression similar to those shown in the bacterial system may be active in the mammalian system. These studies are therefore providing a means for understanding regulation of endogenous viral expression in mammalian cell systems as well as defining the mechanism(s) of TPA action.

Proposed Course:

We propose to characterize the suppressor component(s) utilizing biochemical and biophysical techniques. Additionally, the significance of

these factors in influencing tumor promoter effects, particularly on cells in vitro will be investigated. The contributions of retrovirus induction and protease modulation in tumor promotion will also be examined.

Publications:

Hellman, A., Weislow, O.S., Twardzik, D.R., and Fowler, A.K.: Type C retrovirus activation and possible functions in the normal and tumor bearing host. Cancer Res. 39: 2902-2907, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04807-10 LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunocompetence and Susceptibility of Animal Systems to Oncogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. K. Fowler	Head, Bio-molecular Oncology Section	LCMB NCI
Other:	A. Hellman	Assistant Chief	LCMB NCI
	D. R. Twardzik	Biochemist	LCMB NCI
	P. T. Allen	Microbiologist	LCMB NCI

COOPERATING UNITS (if any)	Wright State University School of Medicine Microbiology & Immunology Program Dayton, OH 45435	Litton Bionetics, Inc. Fred. Cancer Res. Ctr. Frederick, Maryland 21701
LAB/BRANCH	Carcinogenesis Intramural Program Laboratory of Cellular and Molecular Biology	
SECTION	Bio-molecular Oncology Section	
INSTITUTE AND LOCATION	DCCP, NCI, NIH Frederick, Maryland 21701	

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.9	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The influence of host physiology on type C retrovirus expression in the normal and tumor-bearing host is being examined. Activation of retrovirus information during pregnancy and by estrogens in reproductive tissues of mice has been demonstrated by several methods including molecular hybridization, electron microscopy, radioimmunoassay and RNA-directed DNA polymerase assay. Further, a component has been extracted from the placenta, and to a lesser extent from the gravid uterus, that exhibits antiviral activity which has biophysical, biochemical and immunological characteristics of virus induced (Type 1) interferon. These results are consistent with our hypothesis that retroviruses participate in normal physiologic processes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The objectives of this project are to develop and examine model systems for: (1) defining and characterizing physiological and immunological factors that modify the susceptibility of the host to tumorigenesis and the mechanism by which oncornavirus contribute to this process, and (2) examining and characterizing oncornavirus x host cell interactions and studing the significance of these interactions in cellular differentiation and recognition phenomena.

Methods Employed:

Oncornavirus expression is determined by radioimmunoassay, molecular hybridization, immunofluorescence, gel electrophoresis, RNA-directed DNA polymerase activity and tissue culture techniques. Interferons are assayed by their antiviral (encephalomyocarditis virus, strain MM) effect on L929 cells. These assays are used in combination with a variety of *in vivo* and *in vitro* parameters of host immunocompetence to assess the influence of oncornavirus on cellular recognition and differentiation processes. These include the *in vivo* maternal x conceptus interaction and *in vitro* one-way and two-way mixed lymphocyte reactions, as well as the response of lymphocytes to specific and non-specific antigenic stimulation.

Major Findings:

Kinetic studies using hybridization with complementary DNA's synthesized from high molecular weight type C viral RNA's have shown that virus-specific sequences were among the earliest non-ribosomal RNA's to accumulate in the uteri of NIH Swiss mice after estrogen treatment. The hybrids formed with R-MuLV cDNA had T_m values 3°-6°C lower than the hybrids with purified viral RNA's and indicate an approximate 2-4% nucleotide sequence mismatching. Following hormone treatment of ovariectomized animals significant increases above the low level of viral-specific RNA continuously present in the uterus (∿0.0005% of cytoplasmic RNA) were observed as early as 4 hrs (3- to 4-fold), with maximum levels at 8 hrs (∿20-fold). Neither total uterine DNA nor total RNA was significantly different from control levels at either 4 or 8 hrs. Subsequently, there was a decrease in the relative concentration of viral RNA; however, levels were still higher than basal at 24 hrs. The increase in viral RNA occurred before a detectable increase in protein synthesis, suggesting that this viral-specific RNA is probably mRNA. This interpretation is consistent with our previous observations that viral p30 and RNA-directed DNA polymerase activity increases in uteri of ovariectomized mice 10 to 12 hrs after estrogen treatment.

Extracts from reproductive tissues at various times during gestation have been examined for antiviral activity by the reduction of encephalomyocarditis virus (Strain MM) induced plague formation in L929 cells. Initial studies revealed the presence of significant antiviral activity (PR_{50}=2500 units/ml)

in mouse term placental extracts. This activity was sensitive to treatment with trypsin, actinomycin D and heat, and was neutralized by anti-interferon gamma globulin. Also, it was not inactivated by low pH treatment (pH 2) and was not removed by high speed centrifugation. By these criteria, the placental component exhibits properties of viral induced (Type 1) interferon. During gestation, interferon was detected in most uterine specimens between 10-15 days after mating; however, samples taken before or after this interval of pregnancy, as well as from virgin mice, had no detectable titer (<10 units/ml). The transient appearance of interferon in the gravid uterus developed abruptly and was highest at the 13-day stage (∿300 units/ml). In comparison, interferon was found in all placental samples and its level increased significantly during gestation (>2500 units/ml). Embryonic and fetal specimens had little antiviral activity.

In continuing studies the active region of R-MuLV gp71 involved in its binding to specific cellular receptors on murine lymphoid cells has been associated with a fragment of less than 30,000 daltons (SDS-PAGE analysis). This enzymatically generated fragment contains nine of the fifteen major tryptic peptides of gp71 and all three glycopeptides associated with the native molecule appear to be contained within this component. The fragment exhibits cellular binding kinetics similar to gp71 except that the divalent cation requirements for optimal binding are less stringent. For gp71 cellular binding there is a high requirement for Ca^{2+} which in part, is replaced by either Mn^{2+} or Cd^{2+} but not by Mg^{2+} or Zn^{2+}. By comparison, substantial binding of the small molecular weight component to cells occurs in the absence of divalent cations and with their addition, binding capacity is only moderately elevated. Chemical and enzymatic methods are being employed to further modify and characterize the active binding region of the viral envelope glycoprotein.

Significance to Biomedical Research and the Program of the Institute:

The essential role of host immunity, as dictated by genetic and environmental factors, in the etiology of some if not all cancers, has become increasingly apparent during recent years. The concept that a modification of natural immunity processes under certain environmental and/or physiological stresses precedes or is concomitant with oncogenic virus expression or infection is not new, although it remains poorly defined. The frequent implication of host physiology and viral expression in the development and pathogenesis of cancer, as well as retrovirus involvement in host immunocompetence, points to the need for further research directed toward elucidating the bio-molecular mechanisms and the degree of retrovirus participation in these phenomena as a prerequisite to the development of means to control human cancers.

Proposed Course:

The cellular membrane receptors for murine ecotropic viruses and the biologically active region of the viral envelope glycoprotein will be further characterized by immunological and biochemical techniques. Additionally, the influence of this legand-receptor interaction on immunocyte regulation and

proliferation will be studied using in vitro radiolabeled techniques. A logical extension of the ecotropic oncornavirus receptor model system, especially as related to the influence of endogenous retroviral expression on the normal and tumor-bearing host, is the identification and characterization of cell surface receptors for xenotropic viruses. These studies are logistically contingent on a good source of the major xenotropic envelope glycoprotein, as well as purification techniques which of necessity must conserve the stringent conformational structure of the native gp70 molecule. The interaction of retrovirus gp70 with cell surface components and its potential modulation of host immune response thereof, will be an invaluable tool for investigating the role of endogenous retrovirus on physiological and immunological parameters in the normal and tumor-bearing host.

Additionally, we intend to pursue our observation of interferon production in reproductive tissues with a view toward correlating its production with the activation of endogenous retroviruses and determining its significance in fetal biology.

Publications:

Fowler, A.K., Reed, C.D., Riggs, C.W., Twardzik, D.R., Weislow, O.S., and Hellman, A.: Depression of Rauscher leukemia virus envelope glycoprotein gp71 binding by lymphoid cells during leukemogenesis in mice. Infect. Immun. 24: 647-655, 1979.

Hellman, A., Fowler, A.K., Twardzik, D.R., Weislow, O.S., and Reed, C.D.: Abolition of lymphocyte blastogenesis by oncornaviral components. Adv. Exp. Med. Biol. 121B: 99-110, 1979.

Twardzik, D.R., Fowler, A.K., Weislow, O.S., Hegamyer, G.A., and Hellman, A.: Cell surface binding proteins for the major envelope glycoprotein of murine leukemia virus. Proc. Exp. Biol. Med. 162: 304-309, 1979.

Hellman, A., Weislow, O.S., Twardzik, D.R., and Fowler, A.K.: Type C retrovirus activation and possible functions in the normal and tumor bearing host. Cancer Res. 39: 2902-2907, 1979.

Fowler, A.K., Reed, C.D., and Giron, D.J.: Identification of an interferon in murine placentas. Nature (in press).

Saviolakis, G.A., Strickland, J.E., Hellman, A., and Fowler, A.K.: Estradiol effect on type C viral gene expression in the uterus of the ovariectomized mouse. Proc. Soc. Exp. Biol. Med. (in press).

Allen, P.T., Strickland, J.E., Fowler, A.K., and Waite, M.R.F.: Antigenic determinants shared by the DNA polymerases of reticuloendotheliosis virus and mammalian type C retroviruses. Virology (in press).

Twardzik, D.R., Reed, C.D., Weislow, O.S., and Fowler, A.K.: The effect of local anesthetics on cell surface receptors for the major envelope glycoprotein of murine leukemia virus. Int. J. Immunopharmacol. (in press).

Twardzik, D.R., Ranchalis, J.E., and Fowler, A.K.: Interaction of murine thymocyte histocompatibility antigens with envelope glycoproteins of Rauscher murine leukemia virus. In Lapin, B. (Ed.): Proceedings of the IXth International Symposium for Comparative Research on Leukemia and Related Diseases. U.S.S.R., Russia Publishing Firm. (in press).

Twardzik, D.R., Ranchalis, J.E., and Fowler, A.K.: Affinity of murine leukemia virus envelope proteins for H-2 gene products. In IXth International Symposium for Comparative Research on Leukemia and Related Diseases. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04831-09-LCMB

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on Reticulum Cell Neoplasm Type B in BALB/c Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ruth M. Merwin	Research Biologist	LCMB NCI
OTHER:	Richard Asofsky	Chief, LMI	LMI NIAID

COOPERATING UNITS (if any)

George Klein and Francis Wiener, Karolinska Institutet, Sweden

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In studies on transplant lines of 50 B lymphocytic neoplasms that arose spontaneously in old BALB/c mice and were previously established as transplant lines in this laboratory, we find that the neoplasms in many instances arise in or home to those organs where cells with the same surface immunoglobulin (Ig) isotype are most common in normal mice. Most IgM tumors homed to the spleen, a number of Ig?D tumors arose in Peyer's patches, most A tumors originated in and homed to gut associated tissue and most G tumors arose and homed to lymph nodes. The M, ?D, or A isotypes, but not the G, are stable on transplantation; that is, they are maintained through many generations of transplant. However, in some instances mice with ?D or A tumor tissue at one site had tumor tissue with either no Ig or with a different isotype at another growth site. We find evidence that tumor tissue growing outside the lymphatic tissues rather than in the spleen, nodes or Peyer's patches have lost one but not necessarily all of the surface Ig isotypes. These tumors can be of value in analyzing the stages of maturation of B lymphocytes and as experimental models of human B lymphocytes in the same stages of maturation.

Project Description

Objectives:

To characterize cell surface immunoglobulin (Ig), karyotype and in vivo growth properties of murine B lymphocytic neoplasms as representing various stages in the differentiation of normal B lymphocytes and for experimental manipulation as models for human B lymphomas.

Major Findings:

1. Fifty spontaneous lymphocytic neoplasms that arose in BALB/c mice and were previously established as transplant lines in vivo in this laboratory were available for study. The site at which the primary tumors arose and the sites where the derivative lines homed and grew are in general where cells with the same surface Ig are located in normal mice. Most IgM tumors home to the spleen, IgA tumors home to the gut associated tissues, some Ig?D tumors arise in Peyer's patches and home to the spleen, and IgG tumors arise in and home mainly to the nodes.

2. Tumors expressing M, A or ?D isotypes on the cell surfaces, but not those with the G isotype, maintained this surface characteristic over many transplant generations. Tumors with the ?D or A isotypes at one site in some instances expressed a different isotype or none at other sites, indicating a capacity for reversible modulation.

3. The capacity to grow outside the lymphatic organs, that is locally at the site of subcutaneous transplant or as ascites after intraperitoneal transplant, was relatively constant for any one tumor line. Most of the IgM lines produced less subcutaneous growth than most lines of other isotypes. Changes with transplantation were almost all in the direction of more subcutaneous growth. This change may be due to a loss of surface Ig as the two IgM tumors that had been tested before the change showed a loss of a surface Ig isotype after the change. Also cells at the subcutaneous site in three lines had no surface Ig as compared to its presence on tumor cells in the spleen. However, a loss of surface Ig did not in all cases result in local growth. A change from growth in the spleen to growth in tissues outside the lymphatic system occurred within a few generations in one line, apparently due to a selection for the local growing cells. The localness could not be reversed by selective transfer of spleen tissue.

Significance to Biomedical Research and the Program of the Institute:

It is known that with each stage in maturation of B lymphocytes there are changes in surface phenotype, in differentiative functions, in replicative rates, in migratory rates and in homing patterns. Any one tumor line provides a large number of cells arrested at a certain stage of differen-

tiation and as such can be used in analyzing the stages in maturation of normal B cells and they can serve as experimental models of human B lymphocyte neoplasms derived from cells in different stages of maturation.

Proposed Course:

1. To determine whether the surface G and A Ig isotypes are synthesized by the cells of the tumors or are extrinsic in origin. The surface Ig will be removed by trypsin and the capacity of the cells to replace it in an Ig-free culture medium will be tested. Also the antigenicity of the IgG and IgA tumors will be tested because antigenicity can lead to the attachment of extrinsic Ig to cell surfaces.

2. The two most common types of human B lymphocytic neoplasms are chronic lymphatic leukemia and malignant lymphoma. Both have IgM on the cell surfaces in most cases and both grow mainly in the nodes at least in early stages of the tumor growth. Therefore, the two lines among the BALB/c tumors with surface IgM that produce node enlargement rather than mainly spleen enlargement are of interest. Their surface markers and growth characteristics will be studied to determine whether they can be considered experimental animal models for the common human B lymphomas.

3. To determine by electron microscopy whether the cells of these tumors contain rough endoplasmic reticulum characteristic of late stages of maturation of B lymphocytes.

4. To continue collaborative studies on the karyotype of early generations of these spontaneous tumors relative to trisomy 15. Studies to date indicate a relatively high incidence of this aberration in the BALB/c spontaneous tumors. In this respect they resemble T cell lymphomas but not B cell lymphomas induced by Moloney, Friend or Abelson viruses.

Publications:

Sanford, K.K., Boone, C.W., Merwin, R.M., Jones, G.M., and Garrison, C.J.: The plate-implant as a bioassay of the neoplastic state of cultured cells. Int. J. Cancer 25: 509-516, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04878-11 LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Attempts to Control a Guinea Pig Leukemia via Immunotherapeutic Approaches

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J.W. Pearson Microbiologist LCMB NCI

COOPERATING UNITS (if any)
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Dr. Nabil Hanna, FCRC, Litton Bionetics, Inc.
D. Ablashi, LCMB, NCI, NIH

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National Cancer Institute, Bethesda, MD 20205

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1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Leukemic guinea pigs put into remission with 125 mg/Kg of cytoxan followed by the administration of admixture of C. parvum (.5 mg) plus x-irradiated blast cells (5×10^7) weekly for 2 weeks and then given vaccine alone at 3 weeks resulted in a 100% increase in MST when compared to animals that received drug alone (MST=49 days). A similar approach utilizing BCG (2×10^7 organisms) in the place of C. parvum resulted in a greater extension in MST when compared to the drug treated group but less efficacious than C. parvum. A soluble antigen extracted from the surface of leukemic blast cells at a dose of 1 mg of protein completely protected animals from a viable syngenic tumor challenge of 2×10^5 cells where doses of .5 and .25 mg's of the antigen afforded 83 and 66% protection, respectively. Interestingly, in vivo and in vitro immunological investigations revealed specific recognition to the soluble antigen preparation.

Project Description

Objectives:

The aim of this project is two-fold: 1) to develop and evaluate control of a guinea pig leukemia (L_2C-EN) as well as other guinea pig neoplasia(s) as they evolve by applying chemotherapy and/or surgery for the destruction or removal of the primary tumor growth followed by the application of "immunomodulators" and specific vaccines alone or in combination to eliminate residual tumor cells and/or prevent metastatic outgrowth and (2) to obtain purified tumor antigens to monitor the immune response of animals following various forms of therapy to determine fluctuations in the immune response of treated animals, to use such soluble antigens for the generation of heterologous antiserum with tumor antigen mono-specificity for use in early detection of relapse and the opportunity to use purified soluble antigen preparations in the form of a vaccine as another modality of therapy.

Methods Employed:

1. Transplantable lymphocytic leukemia and mammary carcinoma in strain 2 guinea pig.

2. The application of chemotherapy, surgery, vaccines and immunomodulators in control of guinea pig neoplasia(s).

3. The preparation of soluble antigen extracts for use in monitoring delayed hypersensitivity response in guinea pig tumor systems.

4. Migration inhibition (MIF), lymphocyte blastogenesis and ^{51}Cr release lymphocytotoxicity assays to monitor the cell-mediated immune response of animals undergoing and/or after immunotherapy.

Major Findings:

Immunotherapy: Studies continued over the last year involving the development and evaluation of effective therapeutic control of a spontaneous lymphoblastic leukemia, L_2C-EN, in the strain 2 guinea pig. Investigations were undertaken to examine various immunostimulators such as C. parvum, BCG, pyran co-polymer and glucan, all of which are presently undergoing clinical trials, to determine their capacity for therapeutic modulation of the immune response of the host toward controlling leukemic cell proliferation. Such studies involved the dose, route and frequency of administration of the stimulators in order to obtain an optimal anti-leukemic effect. Results indicated that only C. parvum and BCG, when given alone were capable of significantly increasing host survival when administered one day after inoculation of (1.5×10^4) viable leukemic cells. The combination of BCG (2×10^7 organisms) or C. parvum (.5 mg) with x-irradiated blast cells (5×10^7) promoted a synergistic response with a significant increase in MST, i.e., 48 and 53 days,

respectively, as compared to a MST of 24 days in the untreated control group. Neither BCG nor C. parvum, with or without x-irradiated blast cells was capable of inducing resistance to leukemic growth when administered on days 4 and 7 post-leukemic inoculation. Therefore, potentiation of the immune response was limited by the progress of the disease at the time of immunotherapy. Earlier studies had revealed that the optimal dose of BCG was 2 x 10^7 organisms given by the intradermal route of administration. An optimal dose for C. parvum was found to be .5 mg when administered in the inguinal axillary areas of the animal. An experiment was then initiated to determine the ability of C. parvum alone or in combination with x-irradiated blast cells to augment host survival against this leukemia following cyto-reductive therapy with a clinically active drug, cytoxan (125 mg/Kg). The administration of C. parvum (.5 mg) weekly for 3 weeks following a cytoxan induced remission period resulted in a MST of 53.0 days as compared to a MST of 49.0 days for the group of animals that received drug alone. However, an enhanced effect was seen when C. parvum (.5 mg) was admixed to x-irradiated blast cells (5 x 10^7) and given at the same time period as for C. parvum alone. In this case, guinea pigs that received the admixture exhibited a MST of 81.0 days. Finally, when the admixture was administered weekly for 2 weeks followed by the vaccine alone at 3 weeks a MST of 97.0 days was obtained in this treated group. Similar investigations utilizing BCG in the place of C. parvum gave a significant increase in MST's as compared to the drug treated alone, however, the overall response was not as good as that observed with C. parvum. These studies are now being confirmed for publication. A tandem study currently completed which involved giving an admixture of C. parvum plus vaccine to animals in a drug induced remission period followed a week later by giving BCG plus vaccine and then vaccine alone the third week revealed an enhancement in death of such treated animals (MST=49.0 days) as compared to the drug treatment alone (59.0 days). Studies are currently underway to ask the question whether such a tandem approach with BCG and C. parvum plus the vaccine could lead to the activation of suppressor "T" lymphocytes, a population of cells known to impede the immune response of the host.

L_2C-EN Soluble Antigen Studies: Biochemical studies were recently undertaken to purify tumor antigens from leukemic blast cells in soluble form for 3 purposes: (1) to monitor the immune response of animals undergoing immunotherapy; (2) to generate a heterologous antiserum with tumor antigen mono-specificity for use in early detection of relapse following a drug induced remission period; and (3) since this spontaneous tumor is weakly antigenic, to purify and concentrate the soluble antigen for use in the form of a vaccine as a part of a therapeutic modality against this leukemia. We have recently shown that a soluble tumor antigen preparation (1 mg) obtained from the surface of blast cells utilizing the lithium diiodosalicylate (LDS) extraction procedure and when emulsified with CFA completely protected guinea pigs when challenged with a 2 x 10^5 viable cell preparation of the syngeneic tumor. In addition, immunizing doses of .5 and .25 mg's of the soluble tumor antigen afforded 83 and 66% protection, respectively, against a similar

viable tumor cell challenge (2×10^5). The specificity of this immune response was demonstrated by the failure of guinea pigs immunized with 1 mg of a soluble antigen extract obtained from the L10 hepatoma ascites tumor to be refractory to a L_2C-EN viable tumor cell challenge. Finally, a cell mediated immune response to the EN soluble antigen was observed in animals based on a positive delayed hypersensitivity response to the soluble antigen 5 weeks post-immunization. Likewise, in vitro testing revealed a specific blastogenic recognition of the soluble antigen by immune leukocytes (manuscript in preparation).

Significance to Biomedical Research and the Program of the Institute:

The significance of this program, to the biomedical research and the program of the Institute is that any therapy leading to effective control of this spontaneous leukemia and/or any other spontaneous guinea pig neoplasia(s) will be submitted for pre-clinical testing, evaluation and eventual clinical trial.

Proposed Course:

Investigations were to continue to focus on new conceptual and technical approaches in the prevention and/or control of a spontaneous lymphatic leukemia, a model that mimics the human counterpart in regards to blood picture, organ involvement, spontaneous etiology and poorly immunogenic surface tumor antigens. A major effort would have been in investigating new modifiers in regards to route, dose and regimen alone or in combination with x-irradiated blast cells or with soluble tumor antigens. Further purification of the EN soluble antigen would have enabled one to monitor the immune response of animals during and/or after the application of immunotherapy as well as the generation of a heterologous antiserum with tumor antigen mono-specificity for use in early detection of relapse, thus allowing one to apply therapy before physical signs of the leukemia. Since the tandem studies utilizing BCG, C. parvum plus the vaccine revealed an enhancement of death, the reported phenomena of the generation of suppressor T cell populations which are known to impede effective immune responses was to be actively investigated. If a population of suppressor cells were to be found during the application of immunotherapy, it would be important to neutralize such an suppressor effect in order to lead to successful immunotherapy.

Publications:

Cowan, F.M., Klein, D.L., Armstrong, G.R., and Pearson, J.W.: Augmentation and inhibition of delayed hypersensitivity by Staphylococcus aureus protein A. Biomedicine 30: 241-244, 1979.

Klein, D.L., Cote, P., Garner, W., Wallen, R.M. and Pearson, J.W.: The use of immunotherapy to enhance tumor rejection immunity in leukemic (L_2C) guinea pigs following remission induction with MeCCNU. Leukemia Res. (in press).

Klein, D.L., Aszalos, A. and Pearson, J.W.: Enhanced macrophage tumoricidal function and T and B blastogenic transformation in mice treated with nystatin. Ann. Rev. Pharmacol. (in press).

Cowan, F.M., Klein, D.L., Armstrong, G.R., Stylos, W.A. and Pearson, J.W.: F_c receptor mediated immune regulations and gene expression. Biomedicine. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04922-14-LCMB

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunoprevention and Immunotherapy of Cancer in Experimental Animals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.J. Huebner	Head, Viral Immunology Section	LCMB	NCI
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	R. Bare	Bio. Lab. Tech.	LCMB	NCI
	R. Trimmer	Bio. Lab. Tech.	LCMB	NCI
	J. Hovis	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any) S. Aaronson, LCMB, NCI; D. Fish and R. Gilden, FCRC; M. Gardner, USC; H. Lynch, Creighton U; C. Bowles, Hazelton Laboratories.

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Viral Immunology Section

INSTITUTE AND LOCATION
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National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	2.0	3.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Tumor cell and viral vaccines successfully prevented transplantable and carcinogen-induced cancers in rats. Passive immunity processed as murine-specific IgG successfully prevented spontaneous leukemias and chemically (3-MC)-induced tumors in mice. Endogenous retroviruses, derived in high titer (10^{11} virus particles per ml) from various strains of mice (AT124, M55, C57L), grown in human and canine cells, and baboon virus (grown in canine and human cells), administered intravenously or intraperitoneally, successfully eliminated existing cancer, including multiple metastatic cancers in dogs and (preliminary) in cats. This radical new approach to viral immunotherapy of a variety of cancers in dogs and cats will be extended to rabbits and primates bearing cancers.

Project Description

Objectives:

A major objective is the production of larger quantities of the retroviral vaccines in the highest possible titers for use in mice, rats, cats and dogs to treat advanced or terminal cancers.

Major Findings:

The virus can be given intravenously and/or intraperitoneally. When administered very slowly, fairly large amounts of virus can be safely tolerated. Most reactions consist of moderately increased temperature, hyperventilation and occasional stress reactions. One dog with widely disseminated lymphosarcoma, when given 3 ml of baboon virus intravenously, was found to be free of all lesions, in effect cured, as confirmed by an exhaustive autopsy by three pathologists after the dog was euthanized nine days after treatment. Several other dogs and one cat, given retrovirus injections, improved sufficiently to be sent home. Some of these animals have been given multiple retroviral injections and in most instances are much improved with good prospects for cure. The battery of retroviruses used, each chosen for its capacity to grow to high titer in the host cancer, leads to rejection of the virus and the cancer cell. As a result, a significant added benefit of the retroviral immunotherapy is that the treated animal continues to express a profound immunity to new cancers. The treatment is simple and, when administered with appropriate care, entirely safe. It can be given subsequent to chemotherapy or other treatments provided they have not totally abrogated the host's immune system.

Proposed Course:

We plan, with our clinical collaborators, some of whom offer experience in chemotherapy and other types of immunotherapy, to develop safe and effective retroviral immunotherapy not only in cancerous dogs and cats, but also in cancerous rabbits and primates, including chimpanzees, with long term follow-up. If and when successful and safe immune protection is demonstrated and confirmed in these species, we will address the use of retroviral vaccines for immunotherapy of human cancer.

Publications:

Fish, D.C., Djurickovic, D.B., and Huebner, R.J.: Prevention of transplantable tumors by adoptive transfer of spleen cells from immunized rats. J. Immunol. 123: 2658-2663, 1979.

Huebner, R.J., Fish, D.C., Demarais, J.T., and Djurickovic, D.B.: Immunosuppression of 3-methylcholanthrene-induced fibrosarcomas in rats preimmunized with endogenous rat retrovirus. Proc. Nat. Acad. Sci. (In press.)

Rhim, J.S., Arnstein, P. and Huebner, R.J.: Chemical transformation of cultured skin fibroblasts from humans genetically predisposed to cancer. The 4th International Symposium on the Prevention and Detection of Cancer. London, 1980. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04924-07-LCMB

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunoprevention of Spontaneous Tumors with Viral and Cellular Vaccines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.J. Kelloff	Medical Researcher	LCMB	NCI
Other:	M. Kende	Expert	LOM	NIDR
	F. Veronese	Visiting Fellow	LCMB	NCI
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SECTION
Viral Immunology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) This project has the long-range goal of prevention by immunological means of naturally occurring or chemically induced tumors in animal model systems that have viral, immunologic and epidemiologic similarities to human tumors. To this end, the project has defined the spontaneous tumor and endogenous type C virus expressions in colonies of untreated aging mice and has measured the host's natural and induced immune responses to same. Certain immunization regimens have been found by this project and other investigators to induce an immune response in the host that is effective in preventing experimentally-induced tumors and type C virus expressions. Therefore, the major emphasis of the project now is to specifically define the effector immune responses that occur and are responsible for the successful prevention of tumors, whether naturally occurring or induced. To achieve this, additional techniques specifically involving lymphocyte separation and characterization are being established. An equivalent emphasis is being placed on the nature and specificity of the target antigens that these immune responses are directed against, especially with regard to the gene products of the type C virus including the more recently defined transforming proteins.

Project Description

PROJECT A:

Objectives:

1. Characterization of the naturally-occurring cell-mediated and humoral immune responses to the structural and nonstructural gene products of the endogenous type C viruses.

2. Examination of the interactions of the naturally-occurring humoral and cell-mediated immunity to type C virus containing tumor cells in the aging mouse and its implications for natural tumorigenesis.

3. Characterization of the effective immune responses responsible for prevention of endogenous type C virus expression and naturally-occurring tumors in mice.

Methods Employed and Major Findings:

The natural immune response in mice to their endogenous type C viruses involves a complex interaction between cellular and humoral immune mechanisms. The virus-specific immune reactivities are a function of age and appear only subsequent to endogenous virus expression. Cellular immune activity was found to reside in a population of lymphocytes that were characterized as natural killer cells based on their absence of theta surface antigens or immunoglobulin or complement receptors. Cellular and humoral virus-specific immune responses co-occur in the same animal and pretreatment of virus-positive target cells with sera from virus-positive aging mice is capable of partially blocking the cytotoxic activity of reactive lymphocytes. The blocking activity of sera from individual mice increases as a function of age and endogenous virus expression and is highly correlated with the virus-specific complement-dependent cytotoxic activity of these sera. Mouse sera, whether naturally immune or immune as a result of hyperimmunization with type C virus, exhibit blocking activity that can be removed by absorption with purified type C virus or purified viral glycoprotein (gp70) but not by absorption with noninfected syngeneic cells. High titered and highly specific antisera directed against certain individual R-MuLV structural proteins reveal blocking activity. Monospecific antisera to gp70 and p12 exhibited high titered blocking reactivities which are absorbable by the respective purified proteins. Blocking activity of antisera directed against other viral structural proteins could not be excluded with certainty. These findings raise the possibility that immunity in the mouse to endogenous type C virus or virus-infected cells involves competition between serum-blocking activity and natural-killer cell activity and further provides a unique model system for studying the mechanism of action of blocking antisera known to have monospecific reactivity against defined and purifiable transplantation antigens.

Natural humoral cytotoxic antibodies from 13- and 18-month old BALB/c mice showed a virus-specific complement-dependent activity against target cells productively infected with xenotropic, amphotropic or ecotropic type C viruses. The cytotoxic activity was the lowest against ecotropic virus-shedding cells. Serum obtained from mice of less than 12-month old mice had no such reactivity. The cytotoxic activity was the lowest against ecotropic virus-shedding cells. Serum obtained from mice of less than 12-month old mice had no such reactivity. The cytotoxic reactivity was found to reside solely in the immunoglobulin M fraction which yielded reactivity comparable to the unfractionated sera concerning both titer and relative reactivities to the target cells infected with different type C viruses. In hyperimmune mouse serum from individual normal BALB/c mice of 18 months of age revealed that 80-90% of them were cytotoxic against virus nonproducer mink target cells expressing gp70 or gag gene product. Absorption of sera from 18-month old normal BALB/c mice with cells shedding Class II or Class III xenotropic virus, amphotropic virus, Rauscher-MuLV, or Class I murine leukemia virus indicate a closer amphotropic-FMR viral subtype specificity of the natural cytotoxic immune response as compared to the amphotropic-xenotropic or amphotropic-ecotropic specificity. The incidence and the level of measured humoral cytotoxic activity was sustained in the tumor bearing animal up to 28 months of age as compared to the background established in the 18-month old animal. However, in the nontumor-bearing animal, the incidence and level of cytotoxic reactivity decayed rather rapidly with aging. The sustained cytotoxic reactivity of the serum from old-aged tumored mice might be involved in the progression of the tumor.

A naturally occurring cell-mediated immune response to endogenous xenotropic and amphotropic type C virus occurs in the normal aging BALB/c mouse. This activity is first measurable at about 12 months of age, appearing subsequent to antigenic or infectious expression of the endogenous type C viruses. Concomitant with the appearance of virus specific natural killer cell activity, a virus specific humoral immune response appears and is measurable by the host sera's ability to block the natural killer cell activity in in vitro assays. The blocking activity of this sera is removed by absorption with Class II xenotropic virus (X2V) or Rauscher leukemia virus (R-MuLV). Normal aging mice appear to have the blocking activity of their sera exclusively in the IgM fraction; however, sera from hyperimmunized mice and goats revealed that IgG can mediate the blocking activity as well. Unlabeled concentrated R-MuLV or X2 virus was shown in inhibition assays to effectively compete with labeled target cells for the natural killer cell activity. The nature of the target antigens of the natural killer cells was found to be the products of both the env and gag genes, as shown by the complementary blocking activity of anti-R-gp70 and anti-R-p12 and by the activity of NK cells against mutant virus infected target cells producing only env gene products or only gag gene products. An examination of natural killer cell activity and blocking activity was made in normal and spontaneous tumor bearing mice aged 18 to 28 months. Both NK cell activity and blocking activity were found to be sustained in tumored animals up to 28 months of age; however, the blocking activity, and to a somewhat lesser extent, the NK cell activity decayed in aging nontumored animals after 18 to 22 months of age.

The data obtained thus far in this report have led to the initiation of experiments which are ongoing. First, since the blocking activity is high in the tumored animal but drops rapidly in normal aging animals, sequential monthly test bleeds are being performed on large groups of aging mice. A portion of the mice are being killed at fixed time intervals but most of them will be killed when having some evidence of tumor or at a fixed termination date. The large sampling should allow the statistical evaluation of whether the sustained blocking activity precedes or follows the histological evidence of tumor. The NK cell activity is also being examined in each individual animal and while this assay cannot be performed serially on the same animal, its performance at the scheduled sacrifices and in the tumored animals should give evidence of the trend of this activity. Secondly, aging tumored and normal BALB/c and C57/Bl mice are also being examined for their NK cell and humoral activity to a battery of transformed and nontransformed cells to see if these mice have activity to cells containing the various transforming proteins that have thus far been characterized. Thirdly, it would appear that the NK cell immunity in the 18-month old animal is mediating a protective effect in the host. Since NK cells are presumed to be <5% of the lymphocyte population within discrete anatomic compartments, such as the spleen, large pools of reactive NK cells will be obtained from 18-month old animals and concentrated by selective procedures since they have the $Ly5.1^{+}Ig^{-}Th^{y-}$ phenotype. Such preparations will not only have concentrated NK cell activity but should be devoid of suppressor cells, particularly present in spleen preparations. Fourthly, experimental means of diminishing the IgM response responsible for blocking will be examined, and finally, the endogenous type C virus subtype whose expression is most closely correlated with natural tumor expression is still not proven. The finding of FMR type immunoreactivity in the older tumored animals is of great interest and will be explored. Independent of etiologic relationships, its expression in tumors as a potential transplantation antigen is important to define.

PROJECT B:

Objectives:

1. Examination of the cell surface reactivity of the 110,000-120,000 molecular weight polyprotein(s) of the feline sarcoma virus and Abelson leukemia virus in nonproducer transformed cells by antisera reactive with the nonstructural and structural components of these polyproteins.

2. Characterization of the host's cell-mediated and humoral immune responses to syngeneic nonproducer and producer transformed cells containing the 110,000-120,000 molecular weight polyproteins.

3. Immunoepidemiologic characterization of rats bearing tumors produced by various etiologic agents or occurring naturally to determine their immune reactivity with the transforming protein.

4. Production of hybridomas of the nonproducer rat cells to obtain high titered specific antibody to the transforming proteins. Such antibody to be used in diagnostic and therapeutic experimentation.

Methods Employed and Major Findings:

Syngeneic cell lines have been derived from embryonic cells of the inbred Fisher rat, thus providing a syngeneic system whereby the host's immune response could be examined against such cell lines productively and non-productively transformed by certain type C viruses. Cell lines under study include producer and nonproducer cells transformed by FeSV, Abelson LV, M-MSV. These various nonproducer clones differ in the types and levels of the gag structural proteins that are produced, but most lines under study, except certain controls, have the transforming proteins. These cell lines, as well as others carrying mutants of the type C viruses in mink cells, as well as rat cells, have been developed by Dr. John Stephenson. Experiments to date have shown that many of the weanling animals inoculated with non-producer transformed rat cells mount an immune response that results in regression of the tumor. Tumor dose kinetic studies are currently in progress. Initial studies reveal that a lymphocyte mediate cytotoxicity to syngeneic and allogeneic target cells containing the polyprotein is present in some of the inoculated animals. The time course of the CMI as it relates to tumor development and regression is under study. The humoral immune responses in these inoculated animals is under study. Further experimentation with antisera to the transforming protein is necessary to establish whether the protein is immunologically active at the cell surface in viable cells. The cell mediated and humoral immune responses of the rat challenged with syngeneic transformed cells will be examined against a variety of target cells in chromium release cytotoxicity assays. The specificity of the reactivities to the transforming protein in both the Abelson and feline systems will be intensively studied as well as the host response to the type C virus structural proteins present in some of the inocula. Inocula will include producer as well as nonproducer transformed cells to determine which confers the best immune responses to the transforming protein as tested by both in vitro and in vivo assays. The lymphocyte and serum fractions responsible for the activity will be separated and characterized. Individual animals, regressors, and nonregressors will be compared in various immunologic assays for their activity to the transforming proteins. Hybridoma production will be attempted pending results of the planned studies.

PROJECTS A AND B:

Significance to Biomedical Research and the Progress of the Institute:

This effort has contributed significantly to an understanding of the endogenous type C virus relationship with its host and its genetic and epigenetic relationship to the host's naturally occurring tumors. It has aided the

understanding of the host's immune response, natural and induced, to its spontaneous and induced tumors and to the viral gene products ubiquitously expressed in these tumors. Independent of etiologic relationships, these viral gene products appear to be acting as transplantation antigens in the tumors. The availability of transplantation antigens whose structural specificities are defined have heretofore not generally been available which has slowed the progress of tumor transplantation research. With defined tumor transplantation antigens the effector immune responses in the host can now be systematically examined to determine their potential protective or enhancing effect on natural or induced tumors. A clear understanding of these immune mechanisms makes immunoprevention of spontaneously occurring and experimentally induced tumors a realistic goal to pursue.

Proposed Course:

Continuation of the above outlined studies.

Publications:

Kende, M., Hill, R., Dinowitz, M., Stephenson, J.R., and Kelloff, G.J.: Naturally occurring lymphocyte mediated immunity to endogenous type C virus in the mouse. Blocking of the lymphocyte reactivity with antisera to the virus. J. Exp. Med. 149: 358-371, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04925-09-LCMB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of RNA Tumor Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	S.A. Aaronson	Chief, Laboratory of Cellular and Molecular Biolgy	LCMB	NCI
	P.E. Reddy	Visiting Scientist	LCMB	NCI
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COOPERATING UNITS (if any) M. Essex, Harvard Univ., Boston, MA.; F. Noronha, Cornell Univ., Ithaca, N.Y.; L. Crittenden, RPRL, East Lansing, Michigan

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION Animal Virology and Field Studies Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were undertaken to determine the prevalence and natural behavior of type C RNA tumor viruses of the cat and the endogenous retrovirus of guinea pigs. Preliminary sero-epidemiologic survey of cats in India suggested that the incidence of feline leukemia virus infection in these cats is low or undetectable at the level surveyed. A sensitive radioimmunoassay (RIA) for the major structural proteins of the chemically inducible guinea pig endogenous retrovirus (GPV) suggested that GPV expression in guinea pig cells and tissues is not correlated with neoplastic transformation and that GPV is not related to the intracisternal type A particles observed in transplantable guinea pig leukemias. Previously described interspecies immunoassays indicating that distantly related retroviral proteins contain common antigenic determinants has promoted the development of a search and use of monoclonal antibodies capable of recognizing these determinants.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

This Section is concerned with laboratory and field studies on the biology, prevalence, transmission, expression, and characterization of selected RNA tumor viruses and their genomes in natural and experimental model systems by methods involving the use of diverse techniques of virology, immunology, cell biology, and molecular biology.

Major Findings:

Survey of Indian cats for infection with feline leukemia virus.
In a preliminary study to examine the question of whether feline leukemia virus (FeLV) and FeLV-specific antibodies occur in cat populations in parts of the world far removed from the Western Hemisphere, we have performed a sero-epidemiologic survey of cats from three geographic locations in India: Bombay, New Delhi, and Madras. Sera of 134 cats stored at -70°C immediately after collection have been examined for isolatable infectious FeLV and for FeLV p27 antigen by an Elisa test. Sixty-eight samples have been examined for the presence of FeLV p27 by RIA and for FOCMA antibodies. These preliminary studies have given negative results for isolatable infectious and demonstrable p27 antigen and FOCMA antibodies.

Preparation of monoclonal antibodies against feline sarcoma virus (FeSV) specific tumor antigens.
In a collaborative study with Drs. Reddy, Barbacid, and Aaronson, we are attempting to utilize monoclonal antibodies to better elucidate the relationship between virus-specific tumor antigen(s) present in cells transformed by FeSV and the FOCMA antigen. Since the monoclonal antibodies have a high degree of specificity in their reactivities, our approach is to utilize the monoclonal antibody technique to dissect out and clarify the relationships between antigens coded for by FeSV strains and to establish their relationship to the FOCMA antigen believed to be a tumor specific antigen coded for by FeLV.

In the initial experiment we used a nonproducing cell line of BALB/c cells transformed by ST-FeSV as immunogen for immunizing BALB/c mice. Experiments are currently in progress to determine the characteristics of the monoclonal antibody cell lines now being established.

Monoclonal antibodies to retroviral structural proteins.
A variety of interspecies immunoassays have been described by us and others which detect genetically divergent retroviruses. Technical limitations have precluded the routine use of these assays to classify new virus isolates and to detect additional viruses; we have initiated an intensive search for monoclonal antibodies capable of recognizing such interspecies determinants. Preliminary results to date, using squirrel monkey retrovirus

and the endogenous guinea pig virus as immunizing viruses, indicate that interspecies monoclonal antibodies can be identified. Several are currently being more fully characterized and hybridomas are being made on a large scale.

Immunoassays which detect guinea pig virus (GPV) structural antigens. In the past year, a sensitive RIA for GPV gp70 was developed which was used to measure the level of the viral major glycoprotein in normal and malignant guinea pig cell cultures and animal tissues. In contrast to results reported earlier for GPV p26, the viral gp70 does not apparently share antigenic determinants with structural proteins of other retroviruses. No naturally occurring antibody has been detected in normal and leukemic guinea pigs against either p26 or gp70, and virus expression in long-term cell cultures is not correlated with the cell's tumorigenic potential. A series of experiments were carried out which established unequivocally that GPV is immunologically unrelated to the intracisternal virus present in guinea pig leukemia cells.

Significance to Biomedical Research and the Program of the Institute:

Present studies provided important information on the biology, prevalence and natural behavior of RNA tumor viruses of the cat and guinea pig. Although the viruses of feline leukemia-sarcoma complex exist in multiple antigenic types in countries of the Western Hemisphere, our limited studies of cats of remotely situated India suggested that cats of this region suffer a much lower infection rate than that observed in western countries. In present studies we are attempting to use the approach of using monoclonal antibody techniques to better elucidate the antigenic relationships between antigens specified by sarcoma viruses and the much studied FOCMA antigen, believed to be a tumor specific antigen coded for by feline leukemia virus. The development and use of sensitive radioimmunoassay techniques for the major internal polypeptide antigen of the guinea pig endogenous retrovirus gave valuable clues as to its prevalence in tissues of normal and leukemic guinea pigs, and enabled us to establish the antigenic distinctness of this virus from the type A intracisternal particles routinely observed in tissues of guinea pigs with transplantable leukemias. The antigenic relatedness of GPV to other retroviruses established in our previous studies prompted the development and use of monoclonal antibodies capable of detecting many different retroviruses of diverse species. Preliminary evidence has verified the feasibility of this approach.

Proposed Course:

Studies of Indian cats will be continued to determine if feline leukemia virus neutralizing envelope antibodies are present in the sera of these cats. The presence of FeLV and RD-114 viral genomes in a cat fetus tissue will be examined by molecular hybridization technique. Studies to produce and test monoclonal antibodies against tumor specific antigens coded for by three strains of feline sarcoma viruses will continue. A collaborative study is being set up with Dr. F. Noronha of Cornell University to determine

if vaccines composed of live FeLV of low virulence (for cats) or mildy inactivated feline sarcoma virus preparations will confer protection in specific pathogen free cats against challenge with a virulent strain of feline sarcoma virus. Monoclonal antibody producing hybridomas to different retroviruses will continue to be made and tested for interspecies reactivities. Various immunoassays will be used with which new viruses will be identified and classified. Examination by fluorescent and electron microscopic methods presently available in this laboratory for imaging purposes will be combined with monoclonal antibody techniques as very specific and powerful probes to search for and uncover as yet undetected endogenous retroviruses of other species.

Publications:

Sarma, P.S., Edwards, F., Law, M., Skuntz, S.F., and Crittenden, L.B.: Chicken sarcomas and expression of chicken endogenous virus after transplantation of virus-free Rous hamster tumor cells. Proc. Soc. Exp. Biol. Med. 163: 515-519, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04930-09-LCMB

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biology of Natural and Induced Neoplasias

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Arnstein	Veterinary Director	LCMB	NCI
Other:	R. Huebner	Head, Viral Immunology Section	LCMB	NCI
	S. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI
	J. Rhim	Research Microbiologist	LCMB	NCI

COOPERATING UNITS (if any) C. Graham, Internat. Cent. Env. Safety, Holloman AFB, NM; A. Hackett, Peralta Cancer Inst.; R. Gilden and D. Fish, FCRC; L.R. Emmons, Calif. Dept. Health Services; M. Gardner, USC; J. Levy, U. Calif.; W. Nelson-Rees, Naval Bio. Res. Lab., Oakland.

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Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Chimpanzee skin cultures have been established and distributed; their response to viral and chemical carcinogens has been studied and resulting abnormal, possibly preneoplastic lines identified. Similar human cultures have also been established in preparation for chimpanzee tumor vacccine trials.

2. In vivo behavior of recombinant mouse type C viruses has aided in the identification of specific genes essential for infectivity and malignancy.

3. Studies on the maintenance and behavior analysis of human and other xenotransplants in athymic nude mice are continuing.

4. Studies on photochemical treatment of tumor cells and tumor viruses show promise in selective killing of malignant cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. Develop methods for consistent malignant cell production in primate tissue; subsequently produce tumor vaccines and perform vaccine trials in primates and lower species. Ultimately, these approaches may be used in human cancer vaccine development.

2. Participate in studies to determine retrovirus genes coding for malignancy by mouse leukemia viruses in their susceptible hosts (newborn mice).

3. Study transplanted human and selected animal xenografts for malignant behavior, tumor-specific antigen production, malignancy-related genes or possible new viruses.

4. Conduct studies of *in vitro* and *in vivo* photochemical effects on normal and neoplastic tissues and tumor viruses.

Major Findings:

1. Studies on chimpanzee and human "normal" tissues. Chimpanzee and human cells show remarkable similarity in their response to tumor viruses.

A. RNA type C viruses of known transforming potential (e.g., baboon pseudotype Kirsten virus) (Rhim) efficiently infect and produce altered cell foci in normal human and chimpanzee fibroblast cultures. These foci, however, fail to show accelerated growth and enlargement at the expense of "normal background" cells; on the contrary, after their initial induction they tend to regress. Such cultures inoculated into immunotolerant athymic nude mice when exhibiting peaks in altered cell numbers fail to produce neoplastic xenografts.

Chimpanzee skin-derived cultures from six different individuals as well as human fetal fibroblasts derived from four different abortuses have given similar results. In addition to the baboon-Kirsten virus, similar abortive transformants were obtained using simian sarcoma (Wolfe) and gibbon-Kirsten. These viruses are known to produce fully malignant transformation in rat (Kirsten) or marmoset (SSV) cells.

These results suggest a built-in block to fully malignant transformation present in human and chimpanzee cells.

B. A prototype DNA tumor virus coding for malignant transformation, AD12/SV40 hybrid, recommended by R. Huebner, produces stable, highly

abnormal cell cultures from human and chimpanzee tissue. These cultures exhibit the following manifestations associated with neoplasia: (1) accelerated cell growth, (2) piled up foci and high saturation density (i.e., loss of contact inhibition), (3) colony formation in soft agar (loss of anchorage dependence), (4) essentially immortal line establishment.

To date, none of these virus-altered lines have shown true malignant transformation capable of progressive neoplasia by xenograft in tolerant (athymic nude) mice in over 30 tests, or by homograft to chimpanzee original donor in one test. Slow growing nodules, however, have been produced in four tests using these altered lines; karyologically proven chimpanzee cells have been recovered from these nodules after 2-1/2 months on the mice. In addition, these cells have produced intraocular noninvasive tumorlets in the athymic nude mice.

These results may also be a manifestation of the stability of primate "benign" tissue cultures (i.e., resistance or block to fully malignant transformation).

2. Murine type C viruses produced by recombination of known leukemogenic Rauscher and known non-pathogenic xenotropes, fully mapped for genome content, titrated and submitted from Dr. Aaronson's laboratory, are being tested in vivo for tumorigenesis; after inoculation into newborn NIH Swiss, NIH nude and 129J mice, the animals are monitored and sampled periodically. In most instances recombinants predicted infectious and malignant on the basis of genome maps are in fact proving positive in vivo (by virus reisolation and pathogenesis). The leukemogenesis seems to be somewhat strain- and immune status-moderated. All of the results are still preliminary.

3. Xenotransplantation experiments in collaboration with Naval Biomedical Research Laboratory (Dr. Nelson-Rees) and the Peralta Cancer Laboratory (Dr. Hackett) are conducted to study in vivo behavior of characterized human tumor cultures. These studies demonstrate that in vivo behavior in tolerant (athymic nude mouse) hosts appears to be genetically determined and reproducible.

4. Photochemical cell and virus treatments. Collaborative studies in Dr. Emmons' laboratories have provided additional evidence that psoralen and long wave ultraviolet light have pronounced cell-killing capability in tumor cultures and lesser effects on normal cultures, thus making the system very interesting for treatment. The only problem is that long wave ultraviolet penetration is very shallow and will not work below the skin. Other forms of radiation are being investigated. Normal cultures treated sublethally show some permanent cell alterations, but the altered cells always ultimately die out and never undergo malignant transformation. This is of further interest as a safety factor in therapy. Psoralen and ultraviolet treatment of tumor viruses is also studied.

Significance to Biomedical Research and the Program of the Institute:

1. Studies on transformation, carcinogenesis and ultimate vaccine preparation from primate tissue will directly contribute to human cancer immunotherapy and/or prevention.

2. The determination of malignancy-specific genes in rodents will aid in the search for similar sequences in human tumor cells.

3. The behavior of xenografted tumors is an economical method of studying human cancers on a relatively economical (mouse) host.

4. Photochemical treatment of tumor cells, viruses and normal tissue may offer leads towards cancer immunotherapy.

Publications:

Rhim, J.S., and Arnstein, P.: Inhibition of chemical-induced carcinogenesis of canine cells in vitro by infection with mouse xenotropic type C virus. J. Nat. Cancer Int. 64: 345-352, 1980.

Rhim, J.S., Arnstein, P. and Huebner, R.J.: Chemical transformation of cultured skin fibroblasts from humans genetically predisposed to cancer. The 4th International Symposium on the Prevention and Detection of Cancer. London, 1980. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04940-13-LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Viruses in Experimental Oncogenesis and Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.A. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI
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	E. Canaani	Expert	LCMB	NCI
	K. Robbins	Expert	LCMB	NCI
	P. Andersen	Senior Staff Fellow	LCMB	NCI
	K. Cremer	Senior Staff Fellow	LCMB	NCI
	J. Merregaert	Visiting Fellow	LCMB	NCI
	K. Nagao	Visiting Fellow	LCMB	NCI
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COOPERATING UNITS (if any)
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S. Salzberg, LCMB, NCI; R.V. Gilden, FCRC
R. Gallo, LTCB, NCI; J. Greenberger, Harvard Univ.

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SECTION
Molecular Biology Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	1.0	4.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to elucidate the mechanisms of action of tumor viruses and to search for evidence of tumor viruses in humans with the eventual aim of developing rational approaches to prevention of human cancer. Topics of present interest include 1) the biology of endogenous retroviruses, 2) the molecular biology of retrovirus replication and transformation, and 3) the application of knowledge gained from these studies to the search for tumor viruses of humans.

PHS-6040
(Rev. 10-76)

Project No. Z01-CP-04940-13-LCMB

Project Description

Objectives:

1. To study the mechanisms of action of RNA and DNA containing tumor viruses.

2. To apply knowledge gained from experimental systems to search for a viral etiology to human neoplasia and to develop rational approaches to prevention and treatment of human tumors.

Methods Employed:

Standard and developmental techniques in virology, cell biology, immunology and biochemistry.

Major Findings:

1. A cleavage map of the Moloney murine sarcoma viral DNA was constructed and compared with that of a spontaneously occurring deletion mutant. By restriction enzyme analysis, it was shown that a region encompassing over 40% of the viral information was not essential for transformation or rescue of the deletion mutant. The transforming region was further localised by analysis of the transforming activity in tissue culture of isolated restriction fragments of linear double-stranded sarcoma viral DNA. In each case, DNA fragments that retained transforming activity preserved the cell-derived insertion sequences of the viral genome. Moreover, such transformants invariably expressed RNA specific to this region. By these two approaches, it was possible to demonstrate that the transforming region of the viral genome begins very near or within the cell-derived insertion sequences. Thus, the transforming gene of this mammalian sarcoma virus originates from within the mouse cell genome.

2. The unintegrated circular DNA form of Moloney murine sarcoma virus (MSV) has been cloned in bateriophage λ. Discrete deletions in the viral genome were shown to occur during propagation of recombinant phage in Escherichia coli. Heteroduplex and restriction enzyme analyses indicated the deletion of tandemly repeated sequences within certain of the cloned MSV DNA inserts. Cloned MSV DNA was used to prepare a probe composed of its acquired cellular (src) sequences, shown previously to be necessary for MSV transformation. Analysis of EcoRI digests of normal mouse cellular DNA revealed the presence of a single 14-kilobase-pair fragment containing these sequences which lacked contiguity with endogenous type C helper viral information of the same cells. Thus, the sarcoma virus-specific sequences of MSV are represented within the normal mouse genome in a manner analogous to that of a cellular gene.

3. The transforming region of the Moloney-murine sarcoma virus (M-MSV) genome has been sequenced utilizing molecularly cloned viral DNA. This region, extending between 3.6-5.8 kbp from the left end of the molecule, contains the entire cellular insertion (src) sequence as well as helper viral sequences including the large terminal redundancy (LTR). On the viral RNA strand, a long open reading frame, 1224 bases in length, commenced very close to the src-helper viral junction and terminated at a point 58 nucleotides into helper viral sequences to the right of src. A possible acceptor splice signal was detected in helper viral sequences upstream from this open reading frame. On the antiviral RNA strand, several promoter-like sequences, including one within the src region itself, were identified. However, no open reading frame downstream from these promoters was detected in the antiviral RNA strand. The LTR was found to contain promoter-like sequences as well as mRNA capping and polyadenylation signals. In addition, it possessed an 11 base inverted terminal repeat at each end. Thus, the structure of the M-MSV genome with an LTR at each end resembles that of prokaryotic transposable elements.

4. Moloney murine sarcoma virus strain 124 (MSV-124) possesses the ability to transform cells but requires a type-C RNA virus as a helper virus for its propagation as an infectious virus. We inquired whether natural selection during virus replication and transmission would lead to alteration of MSV-124 genetic information. Individual viruses were cloned in nonproducer transformants from an MSV-124 stock that had undergone a single or multiple cycles of infection. Analysis of the ability of individual MSV-124 transformants to express helper viral gag gene products indicated the selection of variants that had lost the capacity to express some or all such MSV-124 coded information. The genomic size of individual MSV-124 variants was determined by electrophoretic and hybridization analysis of unintegrated proviral DNA. Viruses isolated following multiple cycles of infection were shown to contain deletions of up to 35% of the parental MSV-124 genome. Moreover, there was a striking natural selection for such mutants. Thus, the evidence indicates that MSV-124 deletion mutants possessed a significant growth advantage relative to the parental MSV-124 genome.

5. Interspecies antigenic determinants have been used as markers to study the evolution of oncoviruses. By radioimmunologic techniques, it has been possible to detect crossreactivities among the major structural proteins of prototype viruses representing each of the three major Oncovirinae genera. Two different subsets of interspecies antigenic determinants were demonstrated. One encompasses known type B and type D viruses; the other is shared by type D and mammalian type C viruses. Such determinants were also demonstrated in certain as yet unclassified oncoviruses. These findings provide experimental evidence supporting the concept of an evolutionary relationship among the three major oncovirus genera.

6. The relationships between OMC-1, an endogenous oncovirus of owl monkey, and representatives of the three oncoviral genera have been investigated by radioimmunological techniques. The major structural protein of OMC-1 was shown to share antigenic determinants with the corresponding proteins of certain type C viruses of rodent, feline, and cervine origin. It was not possible to demonstrate antigenic cross-reactivity between OMC-1 and endogenous type C viruses of baboons. These findings argue that OMC-1 and baboon endogenous viruses do not represent direct descendants of an ancestor virus that became integrated within primates prior to the divergence of New and Old World species. A close antigenic relationship was established between the major structural proteins of OMC-1, an endogenous virus of deer (deer kidney virus), and avian reticuloendotheliosis viruses. These findings establish OMC-1 and deer kidney virus in the evolutionary lineage that may have led to the generation of avian reticuloendotheliosis virus, a group of oncogenic viruses capable of crossing the interclass barrier between mammals and birds.

7. A retrovirus endogenous to guinea pig cells was earlier shown to be morphologically similar to type B and type D prototype retroviruses. Molecular hybridization techniques were used to show that guinea pig virus nucleotide sequences are endogenous to both domestic (Cavia porcellus) and indigenous (Cavia aperea) guinea pigs, but cannot be detected in the DNA of either other hystricomorph rodents or other mammals tested. Using radioimmunological techniques designed to detect interspecies relationships, the major internal polypeptide of guinea pig virus (p26) was shown to share three different sets of interspecies antigenic determinants with squirrel monkey retrovirus, viper retrovirus, and mouse mammary tumor virus. Thus, guinea pig virus appears to provide an evolutionary link between type B and D retroviruses.

8. An approach toward elucidation of the mechanisms of action of mammalian leukemia viruses has been made by the generation in tissue culture of recombinant viruses between a potent murine leukemia virus (MuLV), Rauscher-MuLV, and an endogenous xenotropic mouse type-C virus, BALB: virus-2, without known malignant potential. Using a double selection system devised to select against the temperature-sensitive (ts) lesion associated with a mutant of Rauscher-MuLV and the xenotropic host range of BALB:virus-2, recombinant viruses were obtained at frequencies ranging from 0.01 to 0.1%. Recombinant viruses were identified on the basis of the type specific antigenic determinants in the translational products of gag (p15, p12, p30, and p10 proteins), pol (reverse transcriptase), and env (gp70 glycoprotein) genes. By this approach, the partial genetic maps of a large number of recombinants were obtained. The fact that p10 of Rauscher-MuLV ts 25, the mutant utilized, was the only protein uniformly lacking in recombinant viruses, localized the lesion inhibiting gag precursor cleavage in this mutant at the carboxy terminus of its gag gene.

   The recombinant viruses demonstrated two host range phenotypes as defined by Fv-1 host cell restriction. In each case, NB-tropic recombinants

possessed the p30 of the Rauscher-MuLV parent, whereas the rest, N-tropic in host range, exhibited BALB:virus-2 p30. Thus, it was possible to assign the site of Fv-1 action at, or closely linked, to the viral p30. The target within the viral genome of a second host restriction was also mapped. A serum factor, previously shown to specifically inactivate xenotropic virus infectivity, was demonstrated to exert its action on the viral env gene product. The system described here allows the generation of specific recombinant genotypes that should be useful in defining those regions of the viral genome involved in leukemogenesis.

9. Mouse leukemia viruses (MuLV) have been reported to induce tumors involving cells within the T lymphocyte lineage. In the present study, striking differences were demonstrated in the target cells for in vivo transformation by two clonal replication-competent type C viruses, Moloney- and Rauscher-MuLV. Moloney-MuLV-induced tumors and lymphoma cell lines exhibited Thy.1 antigen in the absence of detectable Fc or C3 receptors, indicating their T cell origin. Rauscher-MuLV primary tumors and lymphoma cell lines of the same mouse strain, however, invariably exhibited Fc receptors in the absence of Thy.1 antigen, suggesting that these tumors were of the B lymphoid lineage. The pattern of immunoglobulin synthesis by individual Rauscher-MuLV tumor cell lines was determined by both biosynthetic and radioimmunologic techniques. Rauscher-MuLV lymphoma lines invariably expressed immunoglobulin heavy (μ) chain in the absence of detectable light (κ or λ) chains. These findings establish that the target of neoplastic transformation in response to Rauscher-MuLV is an immature cell within the B lymphoid lineage. The demonstration of different target cells for transformation by well characterized clonal strains of mouse leukemia virus should aid in elucidating the mechanisms by which these viruses induce malignancy.

10. There is controversy in the literature concerning the presence in humans of antibodies directed against the envelope glycoproteins of known oncoviruses. In the present report, we show that antibodies capable of precipitating a wide variety of oncoviral glycoproteins can be demonstrated under certain assay conditions. Substances as diverse as normal components of serum, extracts of bacteria, and even nonprotein molecules such as glycogen also shared the oncoviral glycoprotein determinants recognized by normal human sera. It was found that immunoprecipitation of a given viral glycoprotein by human sera was entirely dependent upon the cell in which the virus was grown. Human sera specifically did not recognize glycoproteins purified from oncoviruses grown in human or higher primate cells. These findings not only demonstrate that the antibodies were directed against cellular rather than the virus-coded antigenic determinants but also exclude the possibility that this immune response was elicited as a consequence of oncovirus exposure.

11. A large number of cell lines derived from malignant human neoplasms (21 carcinomas or melanomas and 11 sarcomas or gliomas) and 18 human fibroblast lines were examined for ectopic protein production by RIA.

By conservative criteria, 13 malignant lines produced ectopic CG or its β-subunit (0.5-5.6 pmol/mg cell protein). Four of these lines produced ectopic carcinoembryonic antigen (0.04-0.95), while none produced placental lactogen or α-fetoprotein (<0.10). Six malignant lines produced ectopically the common α-subunit of the glycoprotein hormones, and in 2 (ChaGo lung and Chang liver), the secretion rates (391 and 506 pmol/mg cell protein·24 h) were almost 100 times higher than that of any other ectopic protein. Eight malignant lines produced low levels of PRL (0.1-0.57), and 3 lines produced low levels of LH or its β-subunit (0.1-0.26), but neither was secreted. In contrast, only 2 normal fibroblast lines produced CG, and 1 produced PRL-like activity. None of the malignant or fibroblast lines produced GH or FSH or its β-subunit. Moreover, no line produced cortisol, progesterone, 17α-hydroxyprogesterone, testosterone, or aldosterone. Estrone and estradiol were found in media from 4 malignant lines (1.1-9.4 pmol/mg protein). These data suggest that ectopic protein production is 1) widely prevalent in malignant cells in culture and rare in normal fibroblast cultures, 2) nonrandom, with large amounts of certain proteins but undetectable amounts of others, and 3) underestimated by criteria of serum concentration alone, since certain lines produce low levels or do not secrete. The multiple enzymes required for steroidogenesis are not produced together ectopically, but estrogen production by cells in media containing serum may require only a single aromatase or desulfurylase.

12. Normal and genetic variant strains of nonmalignant human fibroblasts produce in vitro a substance indistinguishable from the recently described "pregnancy-specific" beta-1 glycoprotein (PSβG, PAPP-C, SP_1). Demonstration of this presumed placenta-specific material in a nonplacental site raises questions about its normal physiologic role and use as a cancer marker.

Significance to Biomedical Research and the Program of the Institute:

The systems that are being intensively investigated have provided a much better understanding of the biology and biochemistry of viral transformation. It is felt that a clear understanding of these phenomena will significantly speed progress in the search for a viral etiology of human cancer as well as approaches toward cancer prevention.

Proposed Course:

To continue research already in progress in the following major areas:
(1) mechanisms of action of mammalian sarcoma and leukemia viruses;
(2) induction and regulation of retroviruses in virus-negative cells;
(3) determination of the role of viruses in human neoplasia; and
(4) develop new research areas in model systems that pertain to human disease.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04941-08-LCMB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

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Molecular Biology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to biochemically and immunologically characterize retroviruses in order to understand the mechanisms by which these viruses induce cancer in their natural hosts. The role of these viruses in the etiology of human cancers is also under study. Studies currently in progress are the following: 1) biochemical characterization of replication-defective mammalian transforming viruses; 2) biochemical and immunological characterization of new isolates of retroviruses; 3) search for the presence of retroviral genes and gene products in human tumors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. To biochemically characterize mammalian transforming retroviruses.

2. To biochemically and immunologically characterize new isolates of retroviruses.

3. To determine the mechanisms of oncogenesis by retroviruses in their natural hosts.

4. To determine whether or not retroviruses play a role in the etiology of cancers of higher primates, including humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of tumor viruses and their proteins; molecular hybridization techniques using single-strand specific nuclease and hydroxyapatite chromatography to detect and characterize viral genomes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of cloned DNAs by restriction endonuclease mapping, nucleotide sequencing, and electron microscopy.

Major Findings:

Unintegrated 5.8 kbp closed-circular DNA of Moloney-murine sarcoma virus (MSV) strain 124 was isolated from acutely infected cells. This DNA was cleaved to the genome-length linear form by the restriction endonuclease Hind III and ligated to Hind III-cleaved bacteriophage Charon 21A DNA. The recombinant DNA was packaged in vitro into phage particles and propagated in E. coli DP50SupF. Phages containing MSV-specific nucleotide sequences were identified by using molecular hybridization, and their DNAs were subjected to restriction enzyme and heteroduplex analyses. MSV-specific inserts of 6.4, 5.8, 5.2, 4.0 and 2.0 kilobase pairs (kbp) were observed. Several plaque-purified phages were each found to have different sized inserts (for example, 6.4, 5.8, and 5.2 kbp) and, in some instances, phages containing the smallest inserts were observed to accumulate following growth in E. coli. The 6.4, 5.8, and 5.2 kbp forms were found to differ in their degree of representation of 600 bp direct repeats (present at the termini of genomic MSV DNA). The 4.0 and 2.0 kbp inserts were generated from the larger species during propagation of the phage in bacteria and arose by deletion of a continuous segment of DNA starting in each case at about 700 bp from the 5' end of the cloned MSV molecule. Each DNA form was found to induce morphological transformation of cultured mouse cells. However, transforming virus could only be rescued from cells transfected by the 6.4,

5.8, and 5.2 kbp forms. Hind III cleaves the MSV genome within sequences necessary for transformation, but transformation was obtained with cloned MSV DNA that had not been ligated to the circular form. This suggests that ligation of such molecules occurred within the mouse cell, thus generating biologically active molecules. MSV-124 unintegrated DNA was also cloned in Charon 16A by cutting with Xho I which cleaves in the helper-specific sequences of MSV-124. The MSV-specific DNA (5.8 kbp) cloned in Charon 16A was 10 - 100 times more transfectious than the Hind III cloned molecules.

The cloned MSV DNA was used to prepare probes specific for the helper and sarcoma virus-specific (src) sequences of MSV-124. These probes were then utilized to study the arrangement of helper and src-related sequences in normal mouse DNA. EcoRI, for which MSV DNA is not a substrate, was used to cut high molecular weight DNA isolated from several inbred mouse strains. The DNAs were subjected to gel electrophoresis, Southern blotting, and hybridization with each probe. The helper specific probe hybridized with many DNA fragments. In contrast, a single 14 kbp fragment, that did not coelectrophorese with any of the helper-specific bands, was detected with the src-specific probe. Thus, the src-specific sequences of MSV are derived from the normal mouse genome and are present in a configuration analogous to that of a cellular gene. Furthermore, the arrangement of these sequences is conserved in the DNA of several inbred mouse strains.

MSV DNA cloned in λ was subcloned in a plasmid vector (pBR322) to provide larger quantities of DNA for purposes of isolating MSV mRNAs by affinity chromatography and also for providing more readily probes specific for various regions of the MSV genome (i.e, helper, src, terminal redundancies). In the course of these experiments a sequence present in the src region of MSV was found to function as a promoter for the tetracycline resistance gene of pBR322. Nucleotide sequencing of this region identified a sequence very similar to classic procaryotic promoters. The location of this sequence on the MSV genome suggests a unique mechanism for transformation by MSV.

Significance to Biomedical Research and the Program of the Institute:

The availability of highly sensitive and specific biochemical and immunological probes for retroviral gene products has become increasingly of value in order to both demonstrate the possible etiologic involvement of these viruses in human cancers and to study the role that retroviral gene products may play in normal cellular functions.

Proposed Course:

A detailed analysis of the regions of the MSV genome required for transformation, integration, and rescue by helper virus is now under way by using various restriction enzyme-generated fragments of cloned MSV DNA.

The integrated form of MSV-124 and the MSV-related sequences in normal mouse cells are being cloned in order to gain an understanding of the mechanism of transformation by MSV and also to determine the role in oncogenesis of src-related sequences in normal mouse cellular DNA.

Recombinant DNA techniques are being applied and further developed to study other mammalian transforming viruses and cellular genes involved in transformation in species other than the mouse.

Publications:

Tronick, S.R., Robbins, K.C., Canaani, E., Devare, S.G., Andersen, P.R., and Aaronson, S.A.: Molecular cloning of Moloney murine sarcoma virus: Arrangement of virus-related sequences within the normal mouse genome. Proc. Natl. Acad. Sci. USA 76: 6314-6318, 1979.

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Canaani, E., Tronick, S., Robbins, K., Andersen, P., and Aaronson, S.A.: Cellular origin of the transforming gene of Moloney murine sarcoma virus. Cold Spring Harbor Symposium on Quantitative Biology, 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04942-10-LCMB

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Identification of cellular genes with transforming potential.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Viral Genetics Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The research of the Viral Genetics Section continues to be directed towards studies of type C virus encoded transforming protein. For this purpose, we have used a number of model systems with particular emphasis on Abelson murine leukemia virus (AbLV), and several independently-derived isolates of feline sarcoma virus (FeSV). We have shown such viruses to encode polyproteins of 100,000-170,000 molecular weight containing both structural (p15, p12 and variable extents of p30) and acquired sequence encoded nonstructural components. Characterization of the nonstructural components of polyproteins encoded by AbLV and Gardner FeSV have indicated multiple sites of phosphorylation and tyrosine specific protein kinase-associated activity. The involvement of this enzymatic activity in transformation has been established through the isolation and characterization of transformation-defective mutants. The demonstration of tryptic peptides common to the nonstructural components of polyproteins encoded by independent isolates of FeSV raises the possibility that the frequency of cellular sequences with transforming potential may be relatively limited.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The major emphasis of the research of the Viral Genetics Section is directed towards a determination of mechanisms of neoplastic transformation. For this purpose, the possible use of type C viruses as vectors for cloning cellular genes with transforming function is being explored. In addition, efforts are being made to determine whether proteins, analogous to those encoded by type C transforming viruses, are involved in spontaneous and chemically induced tumors.

Methods Employed:

1. Standard cell culture procedures, microtiter cloning techniques, XC plaque assays and transformation assays.

2. Biochemical and immunologic techniques for isolation and analysis of viral and nonviral proteins, competition radioimmunoassays, immunoprecipitation, tryptic peptide analysis and molecular cloning procedures.

Major Findings:

1. The major translation product of Abelson leukemia virus (AbLV), a 120,000 Mr polyprotein, P120, has been compared to translational products representing the entire Moloney murine leukemia virus (MuLV) genome on the basis of [^{35}S]-methionine tryptic peptide composition. Three methionine containing tryptic peptides present in Moloney $Pr65^{gag}$ were each found to be present in both $Pr75^{gag}$ and in $Pr180^{gag\text{-}pol}$. Of these, one peptide, corresponding to Moloney-MuLV p12, but neither of two p30 specific peptides were shown to be present in AbLV P120. Each of the twelve remaining methionine containing peptides present in AbLV P120 were unique to AbLV P120 and not shared by either Moloney-MuLV $Pr180^{gag\text{-}pol}$ or $Pr82^{env}$.

2. In an effort to determine the functional significance of AbLV P120 a genetic approach was undertaken. For this purpose several transformation-defective (td) mutants of Abelson murine leukemia virus (AbLV) were isolated. Cells nonproductively infected with such mutants exhibited a high degree of growth contact inhibition, failed to form colonies in soft agar, lacked rescuable transforming virus and were equally as susceptible as uninfected control cells to transformation by wild type (wt) AbLV pseudotype virus. In addition, representative td AbLV nonproductively-infected cell clones were found to be nontumorigenic *in vivo*. Biochemical analysis of td mutant AbLV infected clones revealed levels of expression of the major AbLV translational product, P120, and a highly-related 80,000 Mr AbLV encoded protein, P80, at concentrations analogous to those in wt AbLV transformed cells. Although AbLV specific 120,000 Mr polyproteins expressed in td mutant AbLV infected clones were indistinguishable from those in wt AbLV transformed lines with respect to molecular weight and [^{35}S]-methionine tryptic peptide composition,

they differed from wt AbLV P120 in their patterns of post-translational phosphorylation. A previously described AbLV associated protein kinase activity was found to recognize as substrate, a tyrosine specific acceptor site(s) contained within a single tryptic peptide common to both AbLV P120 and P80. In vitro [^{32}P] labeling of this phosphorylation site was reduced to below detectable levels in td mutant nonproductively-infected cell clones. These findings establish that AbLV specific polyproteins and their associated protein kinase activities are involved in AbLV tumorigenesis.

3. Polyproteins encoded by the Gardner and Snyder-Theilen isolates of feline sarcoma virus (FeSV) were analyzed with respect to molecular weight, extent of phosphorylation and tryptic peptide composition. As previously reported, cells nonproductively transformed by the Gardner strain of FeSV express a polyprotein of around 115,000 daltons in molecular weight containing feline leukemia virus (FeLV) p15, p12 and a minor portion of p30. In addition, a major 72,000 dalton possible cleavage product was identified. Snyder-Theilen FeSV transformed cells express a major polyprotein of around 115,000 daltons and a second highly-related 80,000 dalton protein. The p12 structural component of Gardner, but not Snyder-Theilen, FeSV P115 was found to correspond to FeLV subgroup A with respect to immunologic type specificity, a finding consistent with the independent origin of these viruses. Tryptic peptide analysis revealed five methionine-containing peptides specific to the nonstructural portion of Gardner FeSV P115, three of which were also represented in Snyder-Theilen FeSV P115. None of these [^{35}S]-methionine labeled tryptic peptides were present in translational products representative of the complete FeLV subgroup A genome, including Pr180$^{gag\text{-}pol}$, Pr65^{gag} and Pr82^{env}. Similarly, phosphorylated tryptic peptides within the structural (p12) and nonstructural components of Gardner and Snyder-Theilen FeSV P115 were found to be highly related. These findings support the possibility that acquired sequences of two independently-derived isolates of feline sarcoma virus encode structurally related proteins.

4. The expression of a 170,000 molecular weight (Mr) polyprotein in each of several McDonough feline sarcoma virus (FeSV) transformed mink cell clones and one McDonough FeSV transformed rat clone was demonstrated. This polyprotein, designated McDonough FeSV P170, was found to contain feline leukemia virus (FeLV) p15, p12 and p30 immunologic determinants and to share two of its five [^{35}S]-methionine tryptic peptides with FeLV Pr180$^{gag\text{-}pol}$. Both of these peptides are specific to the p30 component of Pr180$^{gag\text{-}pol}$. The remaining McDonough FeSV P170 methionine containing peptides are not represented within either FeLV Pr180$^{gag\text{-}pol}$ or Pr82^{env}. Of interest, of the three peptides specific to the nonstructural component of McDonough FeSV P170, one was also represented in the 115,000 Mr polyproteins encoded by the Gardner and Snyder-Theilen strains of FeSV. These findings raise the possibility that the nonstructural components of polyproteins encoded by each of the three independently-derived feline transforming viruses contain both common and unique regions. Moreover, if the sequences encoding these components are involved in transformation, as appears to be the case, our findings

establish that the position of their insertion with the gag-pol region of the FeLV genome can vary between individual isolates.

5. A 150,000 Mr evolutionary conserved cellular phosphoprotein with binding affinity for FeSV P115 has been identified. This newly-described protein, designated P150, possesses broadly-reactive interspecies antigenic determinants, is expressed in cells of a number of mammalian species including cat, mink, dog, rat, mouse and human, and possesses either intrinsic or associated cyclic nucleotide-independent protein kinase activity. On the basis of immunologic and tryptic peptide analysis, we have been able to exclude the possibility that P150 is encoded by cellular sequences analogous to acquired sequences represented within the FeSV genome. Substrates for the mink cellular P150 associated protein kinase include P150 itself, cellular proteins of around 45,000 and 50,000 Mr as well as a 40,000 Mr protein, the detection of which is restricted to nontransformed cells. Apparently as a result of affinity for FeSV P115, P150 is efficiently incorporated into FeSV pseudotype virions, providing a source for its purification and preparation of antisera for study of its expression in mammalian cells.

6. DNA and RNA sequences related to those of RD114 retrovirus and feline leukemia virus (FeLV) were measured by molecular hybridization in embryonic tissues of the domestic cat. Multiple copies of both RD114- and FeLV-related sequences were detected in the DNA of all embryo tissues examined. Although there was a uniform distribution of about 12-15 copies of the RD114-related genetic sequences and 6-7 copies of the FeLV-related genetic sequences per haploid genome, these sequences were differentially expressed in a tissue-specific manner. Embryo thymus tissues contained relatively high levels of RD114 RNA and p30, whereas the expression of FeLV RNA and p30 was low in this tissue. All specimens of placenta expressed intermediate levels of both RD114 and FeLV RNA, and a fraction of each virus-specific RNA was polyadenylated. The presence of RD-114 RNA in the placenta correlated with the detection of RD114 RNA p30, but the translation of FeLV RNA to the corresponding p30 was not generally detected. Low levels of both RD114 and FeLV RNA were found in embryo spleens and livers, whereas the steady-state concentrations of these sequences were either very low or undetectable in the RNA extracted from whole cat embryos and embryonic kidney and brain tissues. The differential expression of these two retroviral genomes in specific embryo tissues of noninbred randomly selected cats suggests a coordinated but not necessarily an essential relationship with embryogenesis and immunologic development.

7. Two distinct isolates of Friend spleen focus-forming virus (SFFV), differing in their erythroleukemic potential, were studied. These isolates were cloned free of their associated helper viruses and shown to be replication-defective. Both SFFV isolates could be rescued from rat fibroblast nonproducer cell clones with helper virus (F-MuLV$_A$ and F-MuLVp) cloned from the anemia- or polycythemia- inducing Friend virus complex. These rescued viruses induced a rapid proliferative disease associated with the appearance of macroscopic spleen foci and splenomegaly. In addition, each was subject

to regulation by the W, S1 and Fv-2 host gene loci. The two isolates of SFFV could, however, be distinguished by both biological and molecular criteria. Friend SFFVp induced a rapid polycythemia associated with the appearance of large numbers of erythropoietin (EPO)-independent erythroid colony forming cells in the marrow and spleen. In contrast, $SFFV_A$ induced a rapid anemia associated with a progressive decrease in the number of EPO-dependent erythroid colony forming cells in marrow, and a rapid increase in the number of EPO-dependent erythroid colony forming cells in spleen. The nature of the disease induced by the two isolates of SFFV was independent of the Friend helper virus: SFFVp, rescued from a nonproducer cell clone with either $F\text{-}MuLV_A$ or $F\text{-}MuLV_P$, induced a polycythemic transformation, whereas $SFFV_A$, rescued with either $F\text{-}MuLV_A$ or $F\text{-}MuLV_P$, induced an anemic transformation. The two Friend SFFV isolates could also be discriminated on the basis of translational products encoded by their gag and env genes: $SFFV_P$ encoded the amino-terminal gag gene protein, p15, whereas $SFFV_A$ was found to encode the gag gene proteins, p15, p12 and p30. In addition, the SFFV isolates were found to encode nonidentical 55,000 Mr env gene related proteins which were distinguishable by analysis of their methionine-containing tryptic peptides.

Significance to Biomedical Research and the Program of the Institute:

By use of type C viruses as vectors for cloning cellular genes with transforming potential, it should be possible to identify and characterize such genes and determine their possible role in spontaneous and chemically induced tumors of man.

Proposed Course:

Our present studies on mammalian sarcoma virus translational products have raised the possibility that these viruses represent genetic recombinants between leukemia viruses and cellular genes coding for transforming proteins. By identification and isolation of transforming virus-coded translational products, it should be possible to analyze spontaneous and chemically-induced tumors in various model systems for expression of analogous proteins. By this means we will be able to test our proposed model for natural tumorigenesis and should be in a position to develop rational immunologic approaches directed towards tumor prevention.

Publications:

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Sacks, T.L., Hershey, E.J., and Stephenson, J.R.: Abelson murine leukemia virus infected cell lines defective in transformation. Virology 97: 231-240, 1979.

Moldow, C.F., Kauffman, R.S., Devare, S.G., and Stephenson, J.R.: Type C and type D primate retrovirus envelope glycoproteins bind common cellular receptor sites. Virology 98: 373-384, 1979.

Van de Ven, W.J.M., Reynolds, F.H. Jr., Nalewaik, R.P., and Stephenson, J.R.: The nonstructural component of the Abelson murine leukemia virus polyprotein, P120, is encoded by newly acquired genetic sequences. J. Virol. 32: 1041-1045, 1979.

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Stephenson, J.R.: Type C virus structural and transformation specific proteins. In Stephenson, J.R. (Ed.): Molecular Biology of RNA Tumor Viruses. New York, Academic Press, 1980, pp. 245-297.

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Stephenson, J.R., Reynolds, F.H. Jr., and Van de Ven, W.J.M.: Abelson murine leukemia virus: Characterization of a polyprotein containing a phosphorylated component(s) encoded by newly acquired sequences. Archiv. fur Geschwulstforschung 50: 204-214, 1980.

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Reynolds, F.H. Jr., Van de Ven, W.J.M., and Stephenson, J.R.: Mammalian RNA transforming viruses encode phosphoproteins with associated protein kinase activity. In Yohn, D.S., Lapin, B.A., and Blakesler, J.R. (Eds.): Advances in Comparative Leukemia Research. Amsterdam, Elsevier/North Holland Biomedical Press, 1979, In Press.

Reynolds, F.H. Jr., Van de Ven, W.J.M., and Stephenson, J.R.: Feline sarcoma virus polyprotein P115 binds a host phosphoprotein in transformed cells. Nature, In Press.

BOOK:

Stephenson, J.R. (Ed.): Molecular Biology of RNA Tumor Viruses. New York, Academic Press, 1980, 527 pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04950-05-LCMB

PERIOD COVERED: October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on Replication-Defective Mammalian Transforming Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	S.R. Tronick	Microbiologist	LCMB	NCI
	E.P. Reddy	Visiting Scientist	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	E. Canaani	Expert	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI
	P.R. Andersen	Senior Staff Fellow	LCMB	NCI
	K.C. Robbins	Expert	LCMB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH: Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: Division of Cancer Cause and Prevention
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Moloney murine sarcoma virus (MSV) specific unintegrated circular DNA was molecularly cloned in bacteriophage λ. During propagation of cloned recombinant DNA in Escherichia coli, specific deletions of viral information were observed. The heteroduplex mapping and restriction enzyme analysis showed that the deletions in virus specific sequences were primarily due to tandemly arranged redundant sequences. 2. The biochemical and immunological analysis of proteins coded by McDonough (SM), Gardner-Arnstein (GA) and Snyder-Theilen (ST) isolates of feline sarcoma virus revealed that GA- and ST- isolates possess common tryptic fingerprints and shared antigenic determinants. These results indicate that these viruses may have been generated by acquisition of similar subsets of cellular sequences. 3. The major translational product coded by ST-FeSV has been shown to be associated with protein kinase activity which catalyzes phosphorylation of tyrosine residues present in heavy chain of immunoglobulin and itself. The resemblance of this unusual protein kinase activity of FeSV-coded protein with analogous proteins of other mammalian transforming viruses suggests that these viruses may have similar mechanism for transformation.

Project Description

Objectives:

To evaluate role of replication-defective mammalian retroviruses in malignant transformation of normal cells. Identification and mapping of nucleic acid sequences with transforming potential from normal and malignant cells to define their involvement in tumorigenesis. Isolation and characterization of retrovirus-coded translational products to understand molecular mechanism of transformation.

Methods Employed:

Heteroduplex analysis to define structure and origin of sarcoma virus specific nucleic acid sequences and biochemical procedures for characterization of retrovirus-coded translational products to evaluate molecular mechanism of cellular transformation.

Major Findings:

1. Molecular mechanism of deletion of sarcoma virus specific sequences during propagation of cloned recombinant DNA in Escherichia coli. The development of molecular cloning techniques have provided a promising approach for amplification of genetic material. In order to define the fine structure of Moloney mouse sarcoma virus (MSV) and to correlate its origin with the nucleic acid sequences in the cellular genome, supercoiled form of MSV DNA was cloned in bacteriophage λ. During amplification of recombinant DNA in Escherichia coli, specific deletions of MSV sequences were observed. In an attempt to understand the molecular mechanism of generation of these deletions, heteroduplex and restriction enzyme analysis was performed on various size virus specific DNA fragments. The results of such analysis revealed that these deletions are generated by specific cleavage of tandemly repeated sequences within MSV DNA inserts. Identification and characterization of smaller viral DNA inserts in λ-MSV clones derived by specific deletions suggested the existence of additional direct repeats in the MSV genome.

2. Acquisition of similar subsets of cellular sequences during generation of independent isolates of feline sarcoma virus. Accumulating evidence indicates that mammalian replication-defective transforming viruses have been generated by recombination of infectious helper viruses and specific cellular genomic sequences. In order to understand the mechanism of cellular transformation by these retroviruses, identification and characterization of the acquired cellular genes and their translational product(s) has been an important aspect of present research programs. Identification of translational products of three independent isolates of feline sarcoma virus (FeSV) lead to studies on biochemical and immunological characterization of these proteins. The results of such studies showed that sarcoma

virus specific polypeptides from two FeSV isolates share common methionine containing tryptic peptides and immunologically cross-reacting antigenic determinants. These findings suggest the possibility that two independent isolates of FeSV have been generated by acquisition of similar, though not identical, subsets of cellular genomic sequences.

3. Protein kinase associated with feline sarcoma virus-coded translational product has unusual specificity for phosphorylation. The mechanism by which replication defective retroviruses transform normal cells to malignant state is intriguing because of the complexity of regulation of gene expression in mammalian cells. One of the important facets in studies on transforming viruses is to identify the transforming virus gene product and study its biological function(s). Feline sarcoma virus-coded polyproteins offer a promising model system for evaluation of its function. The studies on other mammalian transforming viruses, where the translational product of the sarcoma-specific viral genes have been identified, have shown that a protein kinase activity is associated with these proteins. Similar analysis using FeSV-coded polyproteins revealed that these proteins also have a protein kinase activity associated with them which has unusual specificity of phosphorylating tyrosine. These results, along with those reported for other mammalian viruses suggest that there may exist a common mechanism for genetic alteration of cellular regulation by gene products of transforming viruses.

Significance to Biomedical Research and the Program of the Institute:

1. Studies on molecular cloning of murine sarcoma virus showed that the virus specific sequences in recombinant DNA undergoes specific deletions during its propagation in Escherichia coli. The results of hetero-duplex mapping and restriction enzyme analysis showed that these deletions are primarily due to tandemly arranged redundant sequences. Isolation and characterization of smaller inserts in λ-MSV clones derived by specific deletions suggest existence of non-random mechanisms and indicate the possibility of presence of additional direct repeats in the MSV genome. These results are of importance for our present understanding of the structure of viral genome and its role in cellular transformation.

2. The results of investigations on biochemical and immunological characterization of translational products encoded by three independent feline sarcoma virus isolates have been directed to understand the origin of these viruses and their role in malignant transformation. The results of such studies favor the concept that two of these sarcoma viruses have been generated by acquisition of similar subsets of cellular sequences. These results have implications on the origin of these viruses and their biological significance in transforming function.

3. The studies to explore molecular mechanism of transformation of a normal cell by replication defective retroviruses revealed that the major polyprotein coded by FeSV is associated with a protein kinase activity with unusual specificity for phosphorylation. The presence of similar enzymatic activity associated with other mammalian virus-coded transforming proteins suggests that these viruses may exert their effect by a common mechanism. These findings provide insight for the understanding of the mechanism of transformation and furnish a handle for future research in this field.

Proposed Course:

Studies will be continued to determine the role of translational products of mammalian retroviruses in malignant transformation of normal cells. The major emphasis of these investigations will be to understand molecular mechanism of tumorigenesis by genes and gene-products of replication defective transforming viruses and their cellular analogues.

Publications:

Tronick, S.R., Robbins, K.C., Canaani, E., Devare, S.G., Andersen, P.R., and Aaronson, S.A.: Molecular cloning of Moloney murine sarcoma virus: arrangement of virus-related sequences within the normal mouse genome. Proc. Natl. Acad. Sci. USA 76: 6314-6318, 1979.

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Devare, S.G.: Translational products of type D primate retroviruses. In Yohn, D.S., Lapin, B.A., and Blakeslee, J.R. (Eds.): Advances in Comparative Leukemia Research, 1979. Amsterdam, North Holland Biomedical Press, 1980. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04951-04-LCMB

PERIOD COVERED
October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunological Characterization of Retroviruses by Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Dahlberg	Microbiologist	LCMB	NCI
Other:	S. Aaronson	Chief, Lab. of Cellular and Molecular Biology	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)
None.

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Animal Virology and Field Studies Section

INSTITUTE AND LOCATION
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National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Radioimmunoassays to the major structural protein and major glycoprotein of the endogenous guinea pig retrovirus (GPV) were used to show that GPV expression in guinea pig cells and tissues is not correlated to neoplastic transformation. Previously described interspecies immunoassays indicating that distantly related retroviral proteins contain common antigenic determinants has prompted the development of a search for monoclonal antibodies recognizing these determinants.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. To utilize hybridoma technology to develop monoclonal antibodies useful in detecting interspecies determinants present on distantly related retroviruses. To use such assays in classifying retroviral isolates and screening for new viruses.

2. To characterize the endogenous retrovirus of the guinea pig, and to measure expression of GPV antigens by RIA in normal and malignant tissues, and in cell cultures, and to investigate any role that GPV may play in the neoplastic process in the guinea pig.

3. To continue to explore ultrastructural aspects of virus-cell interactions. The detection and classification of retroviral isolates is of particular interest.

Methods Employed:

Light and electron microscopy, cell culture, virus growth and purification, column chromatography, electrophoresis, radioimmunoassays, and a variety of additional preparative and analytical procedures.

Major Findings:

1. We have previously described several new interspecies radioimmunoassays, utilizing an antiserum to a particular retrovirus, and a purified iodinated structural protein of a different retrovirus. These assays were used to demonstrate that the endogenous guinea pig retrovirus, GPV, is immunologically relatd to squirrel monkey retrovirus (SMRV), viper retrovirus (VRV), and murine mammary tumor virus (MMTV). These results were the first demonstration that GPV was related to other retroviruses, and suggested that GPV may represent an evolutionary link between the type B retrovirus (MMTV) and type D retrovirus (SMRV). These assays, as well as additional interspecies immunoassays described by other members of this laboratory, imply that it should be possible to construct a small battery of assays capable of detecting and classifying essentially all retroviral isolates. Conventional approaches to obtaining the appropriate antisera have been only partially successful. Interspecies titers are generally very low, and sensitivity is usually a serious limitation. To overcome these difficulties, a search for interspecies antibodies utilizing hybridoma technology has recently been initiated. Initial results, using SMRV and GPV as immunizing viruses, have been very encouraging. It was anticipated that hybrid cells producing interspecies antibodies would represent a low percentage of the clones, so initial efforts were concentrated on developing procedures to permit large scale growth and screening of hybridoma clones. At present, the laboratory can comfortably grow and screen 50-100 microtiter plates per week. With an average of 30% of the wells containing clones, this represents a minimum of 1500 clones screened per week.

At this time, several monoclonal antibodies have been identified which apparently recognize interspecies determinants. These antibodies will be characterized as to immunoglobulin class by isoelectric focusing, and as to their specificity by immunoprecipitation of labeled viral and cellular extracts.

2. In the past year, an RIA which detects the major glycoprotein of GPV (gp70) has been developed and utilized to augment the results obtained with a previously described assay for GPV p26. In contrast to GPV p26, the viral gp70 does not appear to share immunological determinants with other retroviruses as measured by the lack of ability of sera to such viruses to bind iodinated gp70. A wide variety of sera from normal and tumor bearing guinea pigs failed to bind iodinated GPV gp70, suggesting that GPV does not readily, if ever, induce antibody production in guinea pigs. When normal and malignant tissues were tested for gp70 content, the L_2C leukemia was the only neoplasm found to contain the viral antigen. This is consistent with earlier results using an RIA for GPV p26.

When tissue culture cells either producing GPV spontaneously or following treatment with halogenated pyrimidines were tested simultaneously for both gp70 and p26 content, it was found that cells containing moderately high levels of p26 contained no detectable gp70. The growth medium from these cultures were pelleted and both the pellets and resultant supernatants were tested for gp70 and p26 content. The virus pellets contained approximately equal quantities of each protein, while the supernatants contained a high level of gp70 and only a trace of p26. This was interpreted to mean that GPV gp70 is synthesized at the cell membrane and is immediately incorporated with virions so that the intracellular pool is so small that it cannot be measured with our RIA.

For many years it has been known that the transplantable L_2C leukemia of the guinea pig contained intracisternal A particles in the tumor cells and high levels of virus particles in leukemia plasma. We showed earlier that the "LC" subline of this leukemia contained GPV as well. Some reports have suggested that GPV may be related to or identical to the intracisternal virus. This question has been clarified by our recent demonstration that certain sublines of L_2C leukemia other than the "LC" line lacked detectable GPV p26 and gp70, while the levels of intracisternal virus and plasma particles were virtually indistinguishable from those present in LC leukemia. These results indicate that GPV is immunologically unrelated to the guinea pig intracisternal virus.

Using both RIAs to measure GPV expression, a series of permanent guinea pig cell lines expressing a varying degree of *in vitro* markers of malignancy (cell and colony morphology, growth in soft agar, karyology, etc.) were tested to determine if virus expression could be correlated to the progression of normal cells to a malignant state. No correlation of spontaneous virus expression with malignancy was found, but there was such a correlation with the passage level of some of the cell lines, as has been shown earlier with endogenous murine viruses.

3. We have continued to characterize virus-cell interactions at the ultrastructural level, particularly with regard to the replication, morphogenesis and classification of retroviruses. Much of this effort consists of short term collaborations with members of this and other laboratories. Such a collaboration permitted the R-loop analysis of a cloned portion of sheep pro α2 collagen, in which 17 introns contained 14.1 kb while the exons contained only 3 kb. It is anticipated that EM analysis of nucleic acids will represent a large share of the EM usage, as molecular cloning continues to grow in importance.

In other areas, two retroviruses have been observed in cultures derived from cocultivating indicator cells with kidney cultures of New World rodents related to the guinea pig. Further characterization of these isolates by biochemical means is underway. In addition, ultrastructural analysis of human leukemia cells following treatment with chemical inducers of differentiation has been carried out. We are interested in determing if TPA, sodium butyrate, and other inducers, cause morphological changes apparent in the EM which can be correlated to markers seen by light microscopy. The principal differences seen to date in HL60 and K562 cells treated with inducers are altered ER, nuclear ratio and shape, and changes in vacualization and secretion granules.

Significance to Biomedical Research and the Program of the Institute:

1. The successful isolation of a set of high titered monoclonal antibodies which might collectively identify all, or nearly all, known retroviruses would be of tremendous practical significance. New isolates could be rapidly screened, identified, and perhaps classified. The sensitivity of these assays should be high enough to detect viral antigens in cell and tissue extracts which previously were intractable to such analysis. Such an approach will aid immensely in sorting out how ubiquitous endogenous retroviruses are, and should aid in their detection in non-laboratory animals, including man.

2. The availability of sensitive immunoassays for GPV structural proteins has permitted an analysis of virus expression in normal and malignant cells and tissues. This was vital because GPV is not apparently infectious for cells of other species, and, therefore, bioassays, have not been available. These assays have shown that GPV expression is not apparently correlated with either in vivo or in vitro transformation, and indicate that the virus is similar to most other inducible and non-inducible endogenous retroviruses with regard to oncogenicity. Nevertheless, these assays are available to test new guinea pig tumors in the event that GPV-like oncogenic viruses occur in the future.

3. The electron microscope is a powerful tool for exploring virus-cell interactions, and, in the past few years, has become essential in the analysis of molecular cloning and recombinant DNA research.

Proposed Course:

1. Hybridomas to different retroviruses will continue to be isolated and tested for interspecies reactivities. Various immunoassays will be established with which new virus isolates can be identified and classified. It is anticipated that the technology of producing hybridomas will be utilized by other members of the laboratory for other interesting antigens, such as DNA virus proteins, tumor specific antigens, and so forth. A variety of new fluorescent and electron microscopic techniques are available for imaging purposes, and when combined with monoclonal antibodies, very specific and powerful probes can be made which can be used in both the light and electron microscopes.

2. Project terminated.

3. Monitoring of cells and tissues for evidence of virus expression will continue. The electron microscope can be useful in verifying that viruses are present when other indications are borderline or immunological tests are not available. Electron microscopy of nucleic acids will receive increased attention as recombinant DNA technology advances.

Publications:

Dahlberg, J.S., Tronick, S.R., and Aaronson, S.A.: Immunological relationships of an endogenous guinea pig retrovirus with prototype mammalian type B and type D retroviruses. J. Virol. 33: 522-530, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04967-05 LCMB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of Genetic and Physiological Factors in Viral Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.T. Allen	Microbiologist	LCMB	NCI
Other:	A. Hellman	Assistant Chief	LCMB	NCI
	A.K. Fowler	Head, Bio-molecular Oncology Section	LCMB	NCI
	D.R. Twardzik	Biochemist	LCMB	NCI
	J.E. Strickland	Chemist	LCMB	NCI

COOPERATING UNITS (if any)

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Litton Bionetics
Frederick Cancer Research Center
Frederick, MD

LAB/BRANCH

Carcinogenesis Intramural Program, Laboratory of Cellular and Molecular Biology

SECTION

Bio-molecular Oncology Section

INSTITUTE AND LOCATION

DCCP, NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The relatedness of the avian reticuloendotheliosis (RE) viruses to mammalian type C retroviruses is under investigation. Replication in mammalian cells of the helper virus (REV-A), present in oncogenic preparations of reticuloendotheliosis virus, has been observed and confirmed by a variety of methods including immunogeldiffusion, gel electrophoresis, peptide mapping, viral interference, oncogene rescue, and immune inhibition of viral DNA polymerases. The active site of the DNA polymerase of REV-A was shown to exhibit an antigenic determinant shared with the DNA polymerases of mammalian type C retroviruses. This determinant is not detectable at the active sites of polymerases from types B or D mammalian retroviruses or avian myeloblastosis virus. These results support the idea of an evolutionary link between RE viruses and type C mammalian viruses.

PHS-6040
(Rev. 10-76)

Project Description

Objective

To develop and study model systems for evaluating the role of physiological and genetic factors as they relate to viral carcinogenesis; and to investigate the interaction of antiviral agents with oncoviruses and the effects of such agents on viral oncogenesis and normal developmental processes.

Methods Employed:

Virus infection of cultured cells is accomplished by inoculation of DEAE-dextran-treated cells with preparations of virus passed through 0.45 μm pore-size filters to remove cellular material. Fluid from inoculated cultures is assayed for sedimentable DNA polymerase activity utilizing a poly (A)·oligo(dT) template to monitor for virus production. Virus from producer-cultures is concentrated by centrifugation or ultrafiltration cassettes and purified by sucrose gradient centrifugation. Virus preparations are examined by SDS polyacrylamide slab gel electrophoresis (SDS PAGE). Viruses are identified by a) comparative two-dimensional mapping of the radioiodinated tryptic peptides of the major structural proteins isolated by excision from SDS PAGE slabs, and b) immunogel diffusion testing. Shared antigenic determinants on active sites of DNA polymerases are demonstrated by exposure of the polymerase to antibodies directed against the purified polymerases of the indicated retroviruses. Interferon assays are done using plaque inhibition, viral RNA synthesis inhibition or viral cytopathogenicity inhibition techniques.

Major Findings:

The reticuloendotheliosis (RE) viruses are a distinct group of retroviruses which are responsible for a variety of naturally occurring acute diseases in several avian species. The prototype of the group, REV, is a complex of a defective transforming virus and its replication-competent helper, REV-A. In contrast to other avian viruses, RE virus-related gene sequences have not been found in uninfected avian tissues indicating little likelihood of their evolution from avian genetic elements. These viruses, however, do bear evidence of relatedness to several mammalian type C retroviruses. Their major core proteins are structurally and antigenically related to those of mammalian viruses with a close serological relationship recently reported to the endogenous virus from a New World monkey. Also, their DNA polymerases resemble those of mammalian type C viruses in molecular weight, subunit composition and cation preference. Furthermore, studies within this project have revealed that a non-oncogenic virus component of REV, can replicate in mammalian cells. The oncogenic component, however, has not yet been propagated except in avian cells. Further study has shown that the virus (termed D-REV) propagated in REV-inoculated dog thymus cells is readily transmissible in dog cells.

The identity of this virus has been explored from the hypothesis that it is REV-A. D-REV was shown to exhibit viral interference against transformation by REV in chick embryo cell cultures. D-REV also has been shown to exhibit helper function in rescuing the oncogenic component from REV-transformed, non-virus producer chicken spleen cells. These studies, in agreement with in vivo data showing indistinguishable pathology by D-REV and REV-A, indicate that D-REV is identical to REV-A. It is concluded that only the helper component of REV may be readily propagated in mammalian cells. It should be noted that infection of avian fibroblasts with REV yields transformed fibroblast lines which produce both the transforming and helper viruses. However, serial passage of REV in fibroblasts selects for the helper component. The bio-molecular environment required for propagation of oncogenic REV thus appears to be restricted to conditions found thus far only in avian lymphoid cells. The ability of RE viruses to replicate in mammalian cells distinguishes them from the avian leukemia/sarcoma viruses which do not replicate in mammalian cells, despite the ability of some of these agents to transform mammalian cells.

D-REV has been used in further studies on the evolutionary linkage between RE viruses and mammalian type C viruses. The observation of antigenic similarity has been extended to the DNA polymerases of viruses of these two groups. Antiserum to the DNA polymerase of spleen necrosis virus, an RE group virus, was shown to inhibit the polymerase activity of reverse transcriptases from REV, D-REV and mammalian type C retroviruses of murine, feline, and primate origin. This antiserum did not inhibit the DNA polymerases from avian myeloblastosis virus (AMV) or from several non-type C mammalian retroviruses. Conversely, antiserum to DNA polymerase of a mammalian type C retrovirus, Rauscher murine leukemia virus, inhibited the polymerases of mammalian type C virus and D-REV, but was ineffective against AMV polymerase.

In studies on antiviral agents, an interferon-like activity has been detected in extracts of murine placenta. The origin of this factor and its role in the pregnant animal are under investigation. Preliminary studies suggest that the factor probably originates in the placenta itself.

Significance to Biomedical Research and the Program of the Institute:

The demonstration that reticuloendotheliosis virus is antigenically related to mammalian type C viruses and is capable of replicating in mammalian cells provides further information regarding the evolutionary development of the retroviruses, suggesting a common progenitor for the viruses of these two groups. The demonstration that these avian retroviruses possess the capability for replication in mammalian tissues raises a question of potential health hazards associated with the handling of REV-infected poultry, and points out the validity of requirements for biohazard containment practices in research with all retroviruses.

Proposed Course:

Major emphasis is anticipated on the studies of interferon-like activity present in the placental tissues of mice. The biochemical nature of the active factor will be further characterized in an effort to determine its relatedness to the known classes of murine interferons. A definition of its role in gestation will be sought. Studies will be done to examine the conditions leading to the elaboration of the biologically active molecule.

It is also proposed that experiments will be done to further examine the question of the replication in mammalian cells of the REV oncogene. To this end mammalian-cell-adapted REV-A variants are being propagated which can be used to rescue the transforming component from non-producer REV-transformed cells for use in transmission studies. Studies on the relatedness of REV to other type C viruses will continue.

Publications:

Allen, P.T., Strickland, J.E., Fowler, A.K., and Waite, M.R.F.: Antigenic determinants shared by the DNA polymerases of reticuloendotheliosis virus and mammalian type C retroviruses. Virology (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04976-03-LCMB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Carcinogenesis of Mammalian Cells in Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Gary M. Jones	Microbiologist	LCMB	NCI
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LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.5	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to determine the mechanisms of "spontaneous" and carcinogen-induced malignant transformation in cultured cells. Emphasis is on the development of culture systems utilizing human epithelial cells to study the interaction of chemical carcinogens and viruses with cellular DNA, to define fundamental cytologic and other characteristics of carcinogenic change, and to provide quantifiable criteria of malignant conversion. The project this year consists of the following four studies:

1. Light-induced chromatid damage in relation to DNA repair and malignant transformation in culture.
2. Differential sensitivity of normal cells and their neoplastic derivatives to the lethal action of hydrogen peroxide.
3. Development of human epithelial cell systems for carcinogenesis studies.
4. Evaluation of in vivo assay techniques for determining the neoplastic state of cultured cells.

PHS-6040
(Rev. 10-76)

Project Description

This project consists of the following studies:

Study 1. Light-induced chromatid damage in relation to DNA repair and malignant transformation in culture.

Objectives:

A single 5- to 20-hour exposure of mouse or human cells to visible (405 nm) light at 37°C was shown previously to cause chromatid breaks and exchanges. This damage results primarily from the intracellular production of clastogenic amounts of hydrogen peroxide and the derivative free hydroxyl radical. After prolonged culture, mouse cells show a significant increase in susceptibility to light-induced chromatid damage that is associated with their "spontaneous" malignant transformation in culture. The increased susceptibility could result from loss or inactivation of defense enzymes for destroying H_2O_2 such as catalase or from loss of DNA repair capacities. The first objective was to compare the catalase activities and DNA repair capacities in nonneoplastic mouse cells and their malignant derivatives.

Normal human diploid fibroblasts, in contrast to mouse cells, have not been reported to transform spontaneously. They also exhibit a finite lifespan in culture. Experiments were designed to test (1) whether age in culture or age of donor from which fibroblasts were derived influenced the susceptibility of human cells to light-induced chromatid damage, and (2) whether skin fibroblasts from cancer prone individuals (XP and AT) or skin fibroblasts after carcinogen-induced neoplastic transformation show the enhanced susceptibility to light-induced chromatid damage observed in mouse cells.

Methods:

The conditions for exposure of cells on coverslips to low-intensity fluorescent light (4.6 W/m^2) have been described. Cells were examined in metaphase. For cell cycle studies, light exposure was for 5 hrs prior to examination (late S-G_2) or for 5 hrs with 15 hrs in the dark for G_1 exposure. This protocol is based on 20 hr cell cycle and labelling experiments supported this assumption. To interpret the cytogenetic damage in terms of DNA strand breaks, we have assumed that the prereplication eukaryotic chromosome contains one continuous DNA double strand. Chromatid breaks at metaphase following light exposure during G_1 would result from DNA strand breaks directly or indirectly from crosslinks or base damage, whereas chromatid breaks following exposure during late S-G_2 would result from single or double strand breaks. We have determined the incidence of light-induced chromatid breaks during G_1 and late S-G_2 periods of the cell cycle in nonneoplastic and malignant derivatives.

Major Findings:

Mouse cell system:

No consistent change in catalase activity with respect to neoplastic state could be established in mouse cells. Both G_1 and late S-G_2 periods were susceptible to light-induced chromatid damage in malignant mouse cells but only G_1 in their normal precursors. Since caffeine inhibits DNA repair, we compared its effects on both normal and malignant derivatives to assess their DNA repair capacities. A 5 hr pulse of caffeine (50 µg/ml) to normal cells during late S-G_2 exposure to light or directly following a 5 hr exposure in G_1 increased the chromatid damage to that in malignant cells exposed without caffeine. Caffeine had little effect on malignant cells. It thus appears that normal mouse cells have a caffeine-sensitive DNA repair mechanism(s) that is absent or defective in the malignant cells. This defect in the malignant cells can explain their increased susceptibility to fluorescent light-induced chromatid breaks.

Human cell system:

Thirteen cell lines from normal skin of different aged donors ranging from fetal to 92 years were assayed. In spite of individual variations, no influence of donor age on light-induced chromatid damage was apparent. The variations within the age groups may be related to individual differences in capacity to repair DNA damage. Further, no increase in susceptibility was observed during serial passage of lines from fetal lung, fetal skin, and skin of a 3-yr old male. In fact, susceptibility decreased in the fetal lung cells during the second half of the lifespan. These results suggest that the limited lifespan of human diploid fibroblasts in culture is not related to increased susceptibility or decreased DNA repair capacity. However, susceptibility increased during serial passage of cell lines derived from cancer-prone xeroderma pigmentosum (XP) and ataxia telangiectasia (AT) individuals.

Human skin fibroblasts transformed by chemical carcinogens that grow as neoplasms in nude mice were compared with their normal controls. The transformed human cells like those of the mouse show a significant increase in susceptibility to light-induced chromatid breaks as compared with their nontransformed controls. As observed with mouse cells, a distinct difference was observed between the normal and transformed cells in their susceptibility to chromatid breaks induced by light exposure during late S-G_2 and to a lesser degree during G_1. Whereas exposure of normal cells during late S-G_2 did not increase the incidence of chromatid breaks as compared with that of shielded cultures, exposure of the malignant or neoplastic cells during this period significantly increased the number of chromatid breaks in all 5 lines examined. These results suggest that some defect in repair mechanisms operative at the late S-G_2 stage of the cell cycle is associated with neoplastic transformation of both mouse and human cells.

Proposed Course:

Studies are in progress to elucidate the defects in DNA repair mechanisms of the human cells that have been transformed by carcinogens in culture and also in cells from individuals genetically predisposed to a high risk of cancer. Our goal is to identify DNA repair defects associated with genetic susceptibility to light-induced chromatid damage and human malignancy.

Significance to Biomedical Research and the Program of the Institute:

In recent years a number of observations strongly implicate DNA damage and deficient repair in the complicated process of malignant transformation. Because of the ubiquity of visible light and its diverse effects on mammalian cells in culture, including mutations, DNA strand breaks, DNA protein crosslinks, chromosome damage, neoplastic transformation in mouse cells, and the production of toxic as well as growth-stimulating substances in culture medium, photosensitization may play an important role in mechanisms of carcinogenesis. Skin cancer, the most prevalent form of cancer in the United States today, has been shown to be associated with exposure to sunlight. Further, the finding of increased susceptibility of both mouse and human neoplastic cells to light-induced chromatid damage during late S-G_2 suggests that at this stage of the cell cycle some DNA repair deficiency characterizes neoplastic cell as compared with their normal controls.

Study 2. Differential sensitivity of normal cells and their neoplastic derivatives to the lethal action of hydrogen peroxide

Objective:

Mouse cells after malignant transformation *in vitro* show an increased susceptibility to fluorescent light-induced chromatid breaks; the breaks result primarily from light-induced intracellular production of hydrogen peroxide (H_2O_2) and the derivative free hydroxyl radical. Such cells are also more susceptible to killing by activated macrophages, a reaction in which H_2O_2 has been implicated as the mediator of cytotoxicity. In view of these observations, we compared the sensitivity of paired normal and neoplastic cells to killing by H_2O_2 using two pairs of mouse and two pairs of human cell lines.

Methods:

Cells plated at 10^3 per T-25 in Dulbecco-Vogt medium with 10% fetal bovine serum (DV), after at least 24 hours, were treated for one hour with H_2O_2 in Earle's saline, and colonies counted after approximately 14 days growth in DV.

Major Findings:

In all tests, the H_2O_2-treated neoplastic cells showed a significant dose-related decrease in percent survivors as compared with their paired nonneoplastic controls at H_2O_2 concentrations ranging from 10^{-6} to $5x10^{-5}$ M. This difference was statistically significant at the higher H_2O_2 doses tested. This increased sensitivity to exogenous H_2O_2 following neoplastic transformation could result from loss of defense enzymes for decomposing H_2O_2 or from deficient DNA repair capacities. Our experiments to date with mouse cells (see Study 1) indicate a defect in DNA repair.

Proposed Course:

Further examination of paired neoplastic and normal control human cells as well as cells from individuals genetically predisposed to cancer will be pursued. Activity of the defense enzyme, glutathione peroxidase, will also be measured in these paired cells.

Significance to Biomedical Research and the Program of the Institute:

The development of sensitive and reliable methods for discriminating between cancer cells and normal cells in culture not only facilitates studies of *in vitro* carcinogens but also provides insight into basic cellular lesions in the progression to malignancy. Such insights can stimulate new approaches to selective therapy *in vivo*. In view of the fact that H_2O_2 is a normal cellular metabolite, the handling of excess amounts introduced exogenously may be critical in determining whether the genetic material is damaged. These findings suggest that the inability to destroy this product or to repair the clastogenic effects on DNA may be an important feature of malignant cells of both mouse and human origin.

Study 3. Development of human epithelial cell systems for carcinogenesis studies (see also report of Dr. W. Taylor, Z01-CP-04978-03-LCMB).

Objectives:

1. To develop techniques for culturing normal human epithelial cells for use in carcinogenesis studies.

2. To develop a strategy for transforming human skin epithelial cells with physical and chemical environmental agents.

Major Findings and Accomplishments:

1. A new culture medium formulation for growth of human skin epithelial cells has been published.

2. A manuscript by Camalier, Gantt et al., "Effect of visible light on the cytotoxicity and DNA-binding of benzo(α)pyrene in cultured human skin epithelial cells" has been submitted for publication for which large numbers of explant cultures were made available.

3. With the new medium and the development of specialized techniques, it is now possible to initiate viable replicate cultures from cell suspensions prepared directly from foreskin. These are being used to quantify cell responses to various components of the medium, carcinogens, and physical environmental agents.

Proposed Course:

With these recent technologic advances and gentle methods of synchrony, we are currently developing methods for increasing the fraction of cells in S phase on the premise that the initial events in carcinogenesis occur during replication and replication repair of DNA. Further use of these cultures in collaborative virus studies with other members of this laboratory and other laboratories will be continued.

Significance to Biomedical Research and the Program of the Institute:

Since most human tumors are carcinomas of epithelial cell origin, it is important to develop an in vitro model of carcinogenesis with this cell type. Skin epithelium was selected because of the availability of foreskin and because skin cancer is one of the most prevalent forms of cancer in the United States today.

Study 4. Evaluation of in vivo assay techniques for determining the neoplastic state of cultured cells.

Objective:

To evaluate bioassay techniques for the neoplastic state of cells in culture.

Method:

The implantation of mouse cells attached to polycarbonate plates or glass helices was compared with the subcutaneous or intramuscular injection of comparable numbers of cells in suspension or the intraocular injection of 10^5 cells into syngeneic hosts.

Major Findings:

A total of ten lines or clones were tested on plates. Of four lines that were nontumorigenic when inoculated in suspension either subcutaneously or intraocularly, two were tumorigenic when implanted subcutaneously attached to plastic plates. The remaining two are the first permanent rodent lines that have not been tumorigenic on plastic plates. Of the remaining six

lines, all were tumorigenic on plates and two produced sarcomas at a higher frequency on plates than in suspension. Cytologic diagnoses of the cells implanted on plates, subcutaneously, and intraocularly correlated best with the results from plate implants. Of three mouse lines implanted on helices, two were equally tumorigenic in suspension and on helices, whereas the third was tumorigenic on helices only. The plate-implant was more satisfactory technically than the helices and proved to be one of the most sensitive bioassays for the neoplastic state of cultured cells.

Proposed Course:

The plate implant technique will be used for assaying human cells in nude mice as these become available in our carcinogenesis studies.

Significance to Biomedical Research and the Program of the Institute:

For studies on the mechanism of carcinogenesis in vitro or for evaluation of suspect carcinogenic agents on cultured cells, it is essential to know the state of the cells before as well as after experimental treatment, i.e., whether they are normal, partially transformed preneoplastic, fully transformed cancer cells, or mixtures of these cell types. If the target cells are of mouse, rat, Syrian hamster, or Chinese hamster origin, this information is particularly important because cells of these species regularly undergo "spontaneous" malignant neoplastic transformation during culture. When the experimental treatment produces a relatively permanent change or "transformation" of cell properties in vitro, it is also essential to know whether the alteration reflects a malignant neoplastic transformation. For these reasons, a sensitive bioassay procedure is important.

Publications:

Sanford, K.K., Parshad, R., Handleman, S.L., Price, F.M., Gantt, R.R., and Evans, V.J.: Serum-induced chromosome damage and neoplastic transformation of mouse cells in vitro. In Vitro 15: 488-496, 1979.

Sanford, K.K., Parshad, R., Jones, G., Handleman, S., Garrison, C., and Price, F.: Role of photosensitization and oxygen in chromosome stability and "spontaneous" malignant transformation in culture. J. Natl. Cancer Inst. 63: 1245-1255, 1979.

Parshad, R., Sanford, K.K., Tarone, R.E., Jones, G.M., and Baeck, A.E.: Increased susceptibility of mouse cells to fluorescent light-induced chromosome damage after long-term culture and malignant transformation. Cancer Res. 39: 929-933, 1979.

Parshad, R., Sanford, K.K., Taylor, W.G., Tarone, R.E., Jones, G.M., and Baeck, A.E.: Effect of intensity and wavelength of fluorescent light on chromosome damage in cultured mouse cells. Photochemistry and Photobiology 29: 971-975, 1979.

Gantt, R., Jones, G.M., Stephens, E.V., Baeck, A.E., and Sanford, K.K.: Visible light-induced DNA crosslinks in cultured mouse and human cells. Biochim. Biophys. Acta 565: 231-240, 1979.

Price, F.M., Camalier, R.F., Gantt, R., Taylor, W.G., Smith, G.H., and Sanford, K.K.: A new culture medium for human skin epithelial cells. In Vitro 16: 147-158, 1980.

Sanford, K.K., Boone, C.W., Merwin, R.M., Jones, G.M., and Garrison, C.V.: The plate implant as a bioassay for the neoplastic state of cultured cells. Int. J. Cancer (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04977-03-LCMB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Primary Biochemical Alterations Leading to Neoplastic Conversion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.R. Gantt	Research Chemist	LCMB	NCI
OTHER:	K.K. Sanford	Head, In Vitro Carcinogenesis Section	LCMB	NCI
	W.G. Taylor	Research Biologist	LCMB	NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This program is designed to identify primary chemical and structural alterations in DNA leading to neoplastic transformation in human cells and rodent cells and quantitate the repair response of the cells to agents which induce the primary alterations. The immediate objectives are to develop methods to transform human epithelial cells and to compare the type of lesions caused by low level ("nontoxic") insults to DNA with high level (toxic) insults and measure the cell repair response to these lesions. The lesions currently of most interest are DNA-protein crosslinks including both their induction and repair.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To identify primary changes in cellular and viral nucleic acids during photochemical, chemical, and viral carcinogenesis and to develop and apply techniques for assaying the repair responses of the cells. The use of human epithelial cells is emphasized where it is technically feasible and the study of the induction, consequences, and repair of DNA-protein cross-links is currently stressed.

Methods Employed:

The standard laboratory techniques for measuring single and double strand DNA breaks, repair synthesis, base damage, base adducts, and crosslinks are used with the usual adaptations and innovations.

Major Findings:

1. Low intensities of cool-white fluorescent light induce crosslinks in the DNA of both human and mouse cells in culture at normal working light levels (150 foot-candles for 20 hrs) and under normal cell growth conditions. These crosslinks have been shown to be DNA-protein and to be affected by a variety of conditions (described in detail in ref. 1) including oxygen, light intensity and wavelength, etc. Further, they have been shown to repair within 24 hours. This repair is sensitive to caffeine indicating that the cells cope with the damage by means of the post replication repair mechanism. However, excision repair mechanisms are implicated as well since during the repair period in the presence of caffeine prelabeled DNA can be shown to decrease in molecular weight. Conversely, the repair of DNA-protein crosslinks induced by trans-$Pt(11)$ is unaffected by caffeine (manuscript in preparation). These results indicate two classes of cross-links and/or two mechanisms of coping with the crosslinks based on caffeine sensitivity. Preliminary experiments suggest the existence of two mechanisms of repair, one of which is induced by the light treatment.

Cells from individuals with the genetic disorders FA, AT or progeria were tested for susceptibility and capacity to repair light induced DNA crosslinks and all three were found similar to normal cells.

2. Intermittent exposure to low-intensity fluorescent light protects human skin epithelium in culture from the cytotoxicity of benzopyrene. Light exposure also reduces dramatically the covalent binding of benzopyrene to cellular DNA, inactivates 3-hydroxyl benzopyrene hydroxylase, and destroys benzopyrene in the medium. Even though BP is destroyed by light, light also produces covalent binding of BP products to purified DNA under culture conditions. These observations emphasize the importance of controlling the exposure of cultures to visible light during metabolic

and carcinogenesis experiments. Low levels of BP (0.1 µg/ml) bind to cellular DNA independently of light exposure and from stable adducts not removed during a subsequent week of incubation. Conceivably, DNA damage from chronic low level exposure to BP in the environment could accumulate in human epithelial cells to unexpectedly high levels (manuscript submitted).

3. With respect to other carcinogenic agents, the N^2-guanine RNA methyltransferase associated with RNA tumor viruses (RSV, RAV-0, MuSV, MuLV, AMV, and MTV) was purified from AMV and characterized (ref. 2). Though chick embryo cells contain this enzyme, which methylates nucleoside #10 in $tRNA^{phe}$, the molecular weight of the virus associated enzyme is nearly three times larger and the pH optimum is 0.5 units lower. Surprisingly, analysis of intracisternal virus-like A particles revealed at least six different methylases bound to the core particle rather than only the expected N^2-guanine methylase found in all previously examined type C viruses. Using tRNA as a substrate, we found no difference from the cellular enzymes in specificity, and three were shown to be clearly identical (ref. 3).

Significance of #1 and #2:

The general experimental approach is designed to subject cells in culture (human cells when feasible) to insults at comparatively low, chronic levels for longer time periods so that exposure may better simulate the conditions encountered in the environment. In this way, those insults which cells can easily cope with (single strand DNA breaks, for example) are readily repaired, while those which are more difficult for the cell to cope with tend to accumulate to a measurable extent. For example, large doses of cool-white fluorescent light cause many single strand breaks in DNA which mask the few, potentially important, crosslinks, but the crosslinks can be seen later because the single strand breaks are rapidly and apparently completely repaired, allowing measurement of the small number of crosslinks. Similarly, benzo(α)pyrene adducts to DNA are removed by excision repair after cells are treated at higher concentrations but a small fraction appears refractory to excision and these tend to accumulate. The important point is that this approach focuses on specific DNA lesions in culture which are difficult for the cells to repair and tend to accumulate during chronic exposure to insults. In this way we expect to better study the insults detrimental to the genetic information in animal cells caused by chronic exposure to carcinogenic agents.

The current emphasis on the study of protein-DNA crosslinks is mainly for two reasons. First, epigenetic events may play an important role in promotion and/or stepwise progression in carcinogenesis, and these crosslinks are likely inducers of epigenetic effects as well as causing the expected DNA damage. Second, numerous carcinogenic agents including x-rays, light, and various chemicals (benzopyrene, methylmethase sulfonate, AAF, etc.) cause DNA-protein crosslinks as well as their better known characteristic lesions (DNA breaks, thymine dimers, etc.) The significance of these crosslinks is that they are a special class of damage in eukaryotes where the

DNA is generally covered with protein, so a covalently bound protein would be difficult for cells to monitor by the proposed methods of detecting helix distortion or aberrant bases. Thus, the lesion would be "discovered" only when the cell was about to replicate or transcribe the crosslinked region. This suggests an efficient repair system should be present and that comparatively small losses in efficiency would result in cell genetic damage. For these reasons, it is important to assess the effects of protein-DNA crosslinks on cells during mutagenesis and malignant transformation.

Significance of #3:

Aberrant nucleic acid methylation has been implicated in malignant transformation for over a decade. Because of this proposed relationship we looked for nucleic acid methylases in RNA tumor viruses. One specific enzyme, N^2-guanine methylase, was found in each of the RNA tumor viruses examined, but it appears to be of cellular origin. When intracisternal A particles were examined we found at least six enzymes, apparently bound to the core particle, which again appear to be cellular enzymes. Whether these enzymes are important to the virus or simply fortuitously coisolate with the virus is not known. However, it should be noted that there is a precedent for cellular components being related to virus replication. It has been observed that a specific cellular tRNA is intimately associated with the viral 70s RNA of Rous sarcoma virus and avian myeloblastosis virus and that this specific tRNA is an effective primer of viral DNA *in vitro*.

Proposed Course:

A. Evaluate the susceptibility and repair capacity of human epithelial cells to agents which damage DNA and relate this information to conditions that lead to malignant transformation in culture.

B. Establish clearly whether a light inducible repair system which affects trans-$Pt_{(11)}$ caused DNA-protein crosslinks exists.

C. Assess the types of proteins crosslinked to DNA, i.e., histones, acid proteins, other cellular proteins, serum proteins.

D. Simplify the assay procedure and develop alternate modes of crosslink production.

E. Carefully evaluate the capacity of various human cell lines to repair the DNA-protein crosslinks.

Publications:

Gantt, R., Jones, G., Stephens, E.V., Baeck, A.E., and Sanford, K.K.: Visible light-induced DNA crosslinks in cultured mouse and human cells. Biochim. Biophys. Acta 565: 231-240, 1979.

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Sanford, K.K., Parshad, R., Handleman, S.L., Price, F.M., Gantt, R., and Evans, V.J.: Serum-induced chromosome damage and neoplastic transformation of mouse cells in vitro. In Vitro 15: 488-496, 1979.

Price, F.M., Camalier, R.F., Gantt, R., Taylor, W.G., Smith, G.H., and Sanford, K.K.: A new culture medium for human epithelial cells. In Vitro 16: 147-158, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04978-03-LCMB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Growth, Nutrition, and Neoplastic Transformation of Mammalian Cells In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Katherine K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
	Raymond R. Gantt	Research Chemist	LCMB	NCI

COOPERATING UNITS (if any)

R. Parshad, Howard University College of Medicine

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

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National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-term objective of this program is to understand the mechanism(s) of neoplastic transformation in cultured mammalian cells, particularly human cells. Studies described address two intermediate goals: (a) the role of calcium and dissolved oxygen on survival, proliferation, and differentiated function; and (b) the induction of cellular injury after cell exposure to low-intensity visible light. In studies to date, a lowered calcium concentration appears to retard differentiation of human epithelial cells in that squame formation is not observed; unlike the mouse epithelial system, however, proliferation is not enhanced by lowering calcium concentration. In addition, human skin and monkey kidney cells exhibit a critical oxygen requirement for attachment and proliferation, and monkey kidney cells use oxygen more rapidly than fibroblasts at the same cell density. This rapid utilization affects proliferation and functional responses. Visible light causes chromatid damage in normal human fibroblasts. A medium-specific, cell-type specific cell killing is observed when fibroblasts are exposed to light in medium NCTC 168, a response mediated by hydrogen peroxide products during light exposure.

Project Description

STUDY 1. Modulation of epithelial cell survival, proliferation, and differentiated function by calcium and oxygen.

Objectives:

Most human tumors are carcinomas of epithelial cell origin. Unlike rodent epithelial cells and most human fibroblasts, human epithelial cells are challenging to initiate in culture and difficult to maintain as mitotically active cell populations. Recent studies with rodent cells showed that calcium concentration markedly influences the progression from the dividing undifferentiated basal cell to the anucleated, terminally differentiated keratinocyte. Another recent report showed that transformation of low passage human fibroblasts is enhanced by maximizing the S-phase cell population prior to treatment with carcinogen.

From these observations we have devised a strategy for the transformation of human epithelial cells. Phases of this strategy under present investigation are:

(a) To modify or, as necessary, to develop methods (including culture medium) for treatment of cells capable of both proliferation or terminal differentiation.

(b) To manipulate the culture environment in order to maximize the proportion of cells in S-phase prior to treatment. Physical, chemical, and biological transforming agents (e.g., light energy, chemical carcinogens, and viruses, respectively) will be evaluated.

(c) To select and identify transformed cells, if any, after treatment and to measure their ability to repair induced lesions.

Major Findings:

1. The formulation of medium NCTC 168, an improved medium for primary culture of human foreskin epithelial cells, has been published (ref. 2). This medium, unlike other systems currently in use, supports attachment and epithelial cell proliferation from explants or enzymatically-dispersed suspensions without the use of a feeder layer. This medium supports subculture to the sixth passage level without feeder cells and electron-micrographs show the presence of desmosomes and tonofibrils characteristic of keratinocytes in the passaged cells.

2. Viable enzymatically-dispersed suspensions can now be obtained from either the original tissue or for purposes of subculturing. In part, the calcium concentration is a critical variable. In calcium-free NCTC 168 supplemented with chelex-treated serum limited outgrowth occurs and the

untreated cells are polygonal with limited cell-cell contact. At very low calcium concentrations, epithelial cells are morphologically normal but squame formation appears diminished or absent. However, unlike the mouse system, a lowered calcium concentration does not markedly enhance proliferation.

3. Dissolved oxygen concentration is also a critical variable in the proliferation and functional responses of epithelial cells. Earlier studies showed that unlike fibroblasts, primary human skin and rhesus monkey kidney cells rapidly utilized oxygen during logarithmic phase growth, and moreover, dome (hemicyst) formation required an elevated dissolved oxygen concentration (manuscript in preparation). Recent studies have shown that shutdown or cessation of dome formation, a marker of vectoral transport of salts and fluids, is not inhibited by the accumulation of metabolic byproducts when adequate dissolved oxygen is available. In addition, the frequency of dome formation is influenced by the electrolytic composition of the medium and is not dependent on the presence of glucose as an energy or carbon source.

4. Efforts to optimize the proportion of cells in S-phase during treatment and quantify the mitotically active population by autoradiography are in progress. Significant progress has been made in preparing suspensions of epithelial cells which can be used to quantify nutritional and environmental alterations of the culture system.

5. Studies with Dr. Gantt have assessed the response of human foreskin epithelial cells to concomitant treatment with visible light and chemical carcinogens (submitted for publication). See also Dr. Gantt's report No. ZO1-CP-04977-03-LCMB.

Significance to Biomedical Research and the Program of the Institute:

Both the human skin epithelium and monkey kidney cell systems offer the opportunity to study both basic cell biology and the process of carcinogenesis at the cellular and subcellular level. Several of the above studies highlight the fact that much research with human epithelial cells is as yet empirical in that these cells differ from rodent systems, particularly in their limited proliferative capacity. Results during the last three to five months are sufficiently encouraging that efforts to gently synchronize foreskin epithelial cells and quantify the fraction in S-phase are in progress.

Proposed Course:

Studies on the role of calcium, other cations and hormones on differentiation and proliferation of epithelial cells with major emphasis on human epithelial skin cells are planned. The necessary probes and techniques to identify cells altered by mutagens and carcinogens and subsequent repair of DNA damage have been or are also being established. Collaborative studies with other investigators within the intramural program are in progress or projected as

adequate cultures become available. Continued support for procurement of fresh tissue is essential for the success of these studies. See also Dr. Sanford's report No. Z01-CP-04976-03-LCMB - Study 3.

STUDY 2: Induction of cellular injury or death following exposure of cells to low intensity visible light or light-induced photoproducts.

Objectives:

Visible light and oxygen are ubiquitous environmental agents which interact with both cells in culture and the surrounding milieu. Earlier studies in this laboratory (PNAS 75: 1830-1833, 1978) showed that a 3-20 hour exposure of cells to fluorescent light at an intensity comparable to that in the average laboratory resulted in chromatid damage. Exposure of cell-free culture medium to light results in hydrogen peroxide production, and the addition of catalase to cell cultures during the exposure period significantly reduces the frequency of chromatid breaks and exchanges (ref. 1).

The objectives of these studies were: (a) to determine whether chromosomes of normal human fibroblasts, which remain stable in number, would be sensitive or resistant to light-induced chromatid damage; and (b) to evaluate further the cytocidal influence of light-exposed NCTC 168 for low passage fibroblasts, to determine if this was associated with hydrogen peroxide production, and to test whether one primary event in cell killing was DNA strand breakage.

Major Findings:

1. Exposure of normal Phase II human fibroblasts to low-intensity fluorescent light causes chromatid breaks, which can be prevented by addition of catalase during light exposure. A similar frequency of chromatid breaks is seen when exogenous hydrogen peroxide is added in nonlethal concentrations. Light induced damage can be decreased, but not prevented, by addition of sodium selenite. It is noteworthy that glutathione peroxidase is a selenoenzyme which is induced by sodium selenite and decomposes hydrogen peroxide (submitted for publication). See also Dr. Sanford's report No. Z01-CP-04976-03-LCMB - Study 1.

2. Earlier studies showed that fibroblasts illuminated in NCTC 168 are rapidly killed, whereas primary foreskin epithelial cells appear unaffected. The effective wavelength(s) was narrowed to a range between 365-405 nm, and the cytocidal product shown to be stable in that preilluminated medium killed cells not exposed to light. This killing was largely reversed by catalase, and hydrogen peroxide assays (ref. 3) on illuminated NCTC 168 showed that within 2.5 hours, a lethal concentration, 10^{-4} - 10^{-5} M H_2O_2, is generated. In collaborative studies it was shown that addition of 10^{-4} M H_2O_2 to shielded culture results in frank shattering of cellular DNA, whereas little DNA breakage is observed following a 2 hour exposure of the

same cells to visible light. From this we infer that the primary killing event following illumination in NCTC 168 is not DNA strand breakage, but additional studies to evaluate the relative contribution of crosslinks and DNA breaks are needed to establish the mechanism of killing.

Significance to Biomedical Research and the Program of the Instiute:

Solar energy peaks in the visible range (~ 470 nm), and the radiant energy in the 400-500 nm band is greater than in any subsequent 100 nm band. Skin filters ultraviolet light, but significant amounts of visible light penetrates the superficial layers. Evidence that visible light is mutagenic and promotes neoplastic transformation of otherwise untreated mouse cells is increasing (Cancer Res. 39: 929-933, 1979; Photochem. Photobiol. 31: 135-141, 1980; Science 207: 1209-1211, 1980). Although artificial light is less intense than solar energy, an ever increasing amount of fluorescent lighting is being used.

In tissue culture systems the genome of human cells is damaged by low-intensity fluorescent light, as evidenced by the chromosome studies. Moreover, the cell-type specific killing response in illuminated NCTC 168 (see Study 1) suggests a fundamental biologic difference between those two large categories of cells. It also suggests that visible light-exposure can function as a potentially mutagenic, selective pressure.

Proposed Course:

The present studies raise several new questions. First, are epithelial cells actually refractile to light-induced damage or is the light-induced damage mitigated by the long generation time which permits an adequate time period for repair and/or de novo synthesis, as required? Second, it is of fundamental interest to know how an added enzyme, such as catalase, protects against intercellular chromatid damage. Presumably it is internalized in a phagosome, but this should be established.

Publications:

Parshad, R., Sanford, K.K., Taylor, W.G., Tarone, R.E., Jones, G.M., and Baeck, A.E.: Effect of intensity and wavelength of fluorescent light on chromosome damage in cultured cells. Photochem. Photobiol. 29: 971-975, 1979.

Price, F.M., Camalier, R.F., Gantt, R., Taylor, W.G., Smith, G.M., and Sanford, K.K.: A new culture medium for human skin epithelial cells. In Vitro 16: 147-158, 1980.

Taylor, W.G., Camalier, R.F., and Taylor, M.J.: A spectrophotometric assay for hydrogen peroxide in tissue culture medium. TCA Manual (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04992-03-LCMB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on the Molecular Structure and Function of the Genome of Moloney Murine Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. Canaani	Expert	LCMB	NCI
OTHER:	S.A. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI
	S.R. Tronick	Microbiologist	LCMB	NCI
	K.C. Robbins	Expert	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI
	A. Eva-Varesio	Visiting Fellow	LCMB	NCI
	S. Devare	Visiting Associate	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are: (1) to isolate naturally occurring variants of Moloney murine sarcoma virus (M-MSV) and characterize their biological and structural properties; and (2) study the transforming gene of M-MSV and its transcriptional and translational products.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. By use of recombinant DNA techniques, clone unintegrated circular DNA of M-MSV. Study the cloned DNA for biochemical properties.

2. Characterize the transforming activity of molecularly cloned M-MSV DNA.

3. Isolate naturally occurring variants of M-MSV. Examine their biological properties, identify the segments of M-MSV genome deleted in the variants and characterize their transcriptional and translational products.

Major Findings:

1. Cloned circular viral DNA was isolated from M-MSV infected cells. This DNA was molecularly cloned in bacteriophage λ. A total of five different MSV inserts of 6.4, 5.8, 5.2, 4.0, and 2.0 kbp, respectively, were obtained. Restriction enzyme and heteroduplex analysis indicated that the smaller DNA species were derived by deletion of specific segments of MSV DNA. The deletions were shown to result from the tandem arrangement of the terminal repeats in the cloned molecule, or to be induced by the ends of these terminal repeats.

2. Examination of the biological activity of the molecularly cloned M-MSV DNA molecules showed that they distributed into two classes according to the restriction enzyme site in which they were cloned. Molecules cloned in the Hind III site, which resides within the transforming gene, showed significant but very low focus forming activity. Analysis of the cellular DNA from representative transformants indicated sealing of the Hind III site by integration of two molecules head to tail in tandem. In contrast, MSV DNA cloned in the Xho I site (which lies outside of the transforming gene), was 10-50 times more active in transfection assays. Cellular DNA of the transformant contained M-MSV molecules integrated without rearrangements.

3. A series of nonproducer transformants isolated after infection at limiting dilution with the parental M-MSV-124 stock expressed at least two of the helper viral gag gene-coded proteins (p15 and p12), and in most cases, also p30. Analysis of the viral genomes in these transformants showed them to be of the same size of the parental virus (5800 nucleotides). In striking contrast, none of 20 transformants isolated after infection with an MSV-124 stock that has first undergone multiple cycles of virus infection expressed p30, and more than 80% lacked the ability to synthesize p12 as well. Indeed, the MSV genomes contained in these transformants were all found to suffer various deletions, involving up to 40% of the parental genome. Thus, during the course of multiple cycles of infection by MSV-124, the predominant

population of biologically active virus has undergone a drastic selection towards a smaller genome size. Restriction enzyme analysis of the DNA of one of the mutants further narrowed the region of Moloney-MSV genome essential for transformation. Analysis of viral RNA in nonproducer clones infected with wild type or deletion mutants of M-MSV indicated that all of them contained a subgenomic RNA of 1850 nucleotides. This RNA species is transcribed from the src region as well as from some viral helper sequences in M-MSV genome and might code for the putative transforming protein.

Significance to Biomedical Research and the Program of the Institute:

The molecular characterization of M-MSV transforming gene should help in identifying and characterizing both the putative transforming protein coded by this gene, and the possible related protein coded by the analogous gene in normal cells. This is a unique model system for understanding the conversion of a normal gene to a malignant one.

Proposed Course:

1. Clone the normal cellular gene analogous to M-MSV src gene.

2. Identify transcription and translation signals on this gene.

3. Identify and characterize putative transcriptional and translational products of the normal gene and its M-MSV counterpart.

Publications:

1. Canaani, E., Robbins, K.C., and Aaronson, S.A.: The transforming gene of Moloney murine sarcoma virus. Nature 282: 378-383, 1979.

2. Tronick, S.R., Robbins, K.C., Canaani, E., Devare, S., Andersen, P.R., and Aaronson, S.A.: Molecular cloning of Moloney murine sarcom virus: arrangement of virus-related sequences within the normal mouse genome. Proc. Natl. Acad. Sci. USA 76: 6314-6318, 1979.

3. Canaani, E., Tronick, S., Robbins, K.C., Andersen, P.R., and Aaronson, S.A.: Cellular origin of the transforming gene of Moloney murine sarcoma virus. Cold Spring Harbor Symp. Quant. Biol., 1979, in press.

4. Canaani, E., and Aaronson, S.A.: Naturally occurring deletion mutants of Moloney murine sarcoma virus. Virology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05056-02-LCMB

PERIOD COVERED: October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Molecular Studies of RNA Tumor Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Reddy	Visiting Scientist	LCMB	NCI
Other:	S. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH: Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: Division of Cancer Cause and Prevention
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Molecular organization of Moloney sarcoma virus was studied by nucleotide sequence analysis. The entire transforming region including the terminal redundancies of this viral genome were sequenced. These studies revealed several important biochemical features of this viral genome which enables us to understand the transcription, translation and mechanisms of integration of this viral genome. 2. Abelson-MuLV genome in its integrated form was cloned in E. coli. Biochemical analysis is currently being carried out to understand the structural organization of this viral genome. 3. Studies were carried out to understand cellular targets for in vivo transformation by two clonal replication competent type C viruses--Moloney and Rauscher MuLV. Moloney MuLV was found to induce T-cell tumors while Rauscher MuLV produced tumors of B-cell origin. This provides a model system to understand the mechanisms which are responsible for tissue specificity of these two viruses.

PHS-6040
(Rev. 10-76)

Project Description

PROJECT 1

Objective:

Nucleotide sequence analysis of MSV genome.

Major Findings:

Methods were standardized for nucleotide sequence analysis of DNA molecules. The three methods that were standardized are Sanger's dideoxy method, Maxam and Gilbert's chemical cleavage method and Matt and Smith's nick-translation method. Using a combination of these three techniques the nucleotide sequence analysis of MSV genome cloned in E. coli using λ phage Charon 21A vector by Tronick et al. was undertaken. To the present date, the entire transforming region of Moloney MSV genome as well as the terminal redundancies have been sequenced. The sequence analysis has revealed many important structural features of the genome including the occurrence of promoter-like sequences in the negative strand which suggests the possibility of this strand being transcribed and translated. Also the terminal redundancies exhibit a structural organization similar to prokaryotic transposable elements with "inverted repeats" at the two ends of the redundancy. Sequence analysis of several deletion mutants has revealed that these deletions are mediated by the TRS sequences, further suggesting that these sequences could mediate transposition of the viral sequences.

Currently the sequence analysis of the rest of the sarcoma viral genome is being carried out and a comparison of these sequences with Moloney leukemia viral sequences is being made.

Proposed Course:

The complete nucleotide sequence analysis of MSV genome will be carried out. These sequences will be compared with those of Mo-MuLV and Abelson-MuLV sequences.

PROJECT 2.

Objectives:

1. To study the mechanisms involved in the transformation of B-lymphocytes by Abelson MuLV.

2. To study the effect of such transformation on the development and maturation of B-lymphocytes.

3. To utilize this virus as a tool in developing monoclonal B-lymphocytic cell lines with antigen-binding properties.

4. To utilize such antigen-binding cells to study the mechanisms of antigen-antibody interactions on the cell membrane and to further delineate the molecular mechanisms involved in the maturation of a B-lymphoid cell to a plasma cell.

5. To study if the leukemogenic activity and fibroblast-transforming activity is coded by the same part of viral genome or if two activities are coded by two different regions of the viral genome.

Major Findings:

Our studies to date show that when spleen cells derived from BALB/c mice immunized with SRBC were infected in vitro with Abelson MuLV, a proliferating lymphoid cell culture was obtained in a 4-6 week period. When such cultures were tested for antigen binding activity (SRBC-binding), a varying number of these cells (1-12%) exhibited the antigen-binding activity. Further cloning of these cell cultures in microtiter wells or in soft agar yielded monoclonal cell lines where >90% of the cells had SRBC-binding activity. These cells synthesized small amounts of μ- and κ-chains and the antigen binding activity could be inhibited by treating the cells with anti-mouse μ, anti-mouse κ or anti-mouse Fab antibodies. These cells, when injected into syngeneic mice cause rapidly proliferating lymphoid tumors. These studies demonstrate that Abelson MuLV transforms mature B-lymphoid cells and that monoclonal antigen-binding B-lymphoid cells can be developed using the in vitro transformation potential of this virus.

These antigen-binding B-lymphocytic cultures were then utilized to understand the maturation process of B-lymphocytes to plasma cells. When these SRBC-binding lymphocytic cells were fused with a myeloma cell and selected for hybrid cells, a population of hybrid cells secreting large amounts of anti-SRBC antibody could be obtained. The net increase in antibody production by the hybrid cells is determined as greater than 100-fold. These experiments further demonstrate that transformation of a B-lymphoid cell by Abelson MuLV does not block its ability to maturate into a plasma cell.

To further understand the molecular mechanism of the transforming activity of Abelson-MuLV, attempts were made to clone the viral genome in E. coli using the recombinant DNA techniques. The DNA from mink cells non-productively transformed by Abelson-MuLV was restricted with E.CoRI and the DNA fragments fractionated on an RPC-5 column followed by preparative gel electrophoresis. The viral genome was identified by hybridization with a radioactive cDNA probe made from Moloney MuLV RNA. The purified DNA fragments hybridizing with the cDNA probe were then ligated to λWesB DNA and transfected into E. coli. The clones carrying the viral gene were picked and further purified by replating.

Proposed Course:

The clones carrying the Abelson viral genome will be further characterized by restriction enzyme analysis and nucleotide-sequence analysis. Studies will be conducted to localize the DNA segment carrying the transforming gene. Attempts also will be made to identify the "src" gene present in normal cells and compare its sequence with the "src" gene present in the viral genome. Attempts will also be made to understand what part of the viral genome codes for tissue specificity. Experiments will be conducted to see if fibroblast-transforming activity and leukemogenic activity are coded by the same region of the viral genome or whether these two activities are encoded by two different regions of the viral genome.

PROJECT 3.

Objectives:

To study tissue specificity and mechanisms of leukemogenesis of replication-competent Moloney and Rauscher mouse leukemia viruses.

Major Findings:

Studies were carried out to understand the cellular targets for in vivo transformation by two clonal replication-competent type C viruses--Moloney and Rauscher MuLV. Moloney MuLV induced tumors and lymphoma cell lines exhibited Thy-1 antigen in the absence of detectable Fc and C3 receptors, indicating their T-cell origin. However, Rauscher MuLV primary tumors and lymphoma cell lines of the same mouse strain invariably exhibited Fc receptors in the absence of Thy-1 antigen, suggesting tumors were of the B-lymphoid lineage. The pattern of immunoglobulin synthesis by these various cell lines was determined both by biosynthetic and radioimmunologic techniques. Rauscher MuLV lymphoma cell lines invariably expressed immunoglobulin heavy (μ) chain in the absence of detectable light (κ or λ) chains. All these findings establish that the target of neoplastic transformation in response to Rauscher MuLV is an immature cell within the B-lymphoid cell lineage.

Proposed Course:

Attempts will be made to understand the mechanisms which are responsible for tissue specificity of these two viruses. Attempts also will be made to generate recombinants between Rauscher and Moloney MuLV in an attempt to map the region within the viral genome responsible for tissue specificity.

Publications:

Srinivasan, A., Reddy, E.P., Sarma, P.S., and Schultz, R.A.: Inhibition of RNA-dependent DNA polymerase activity of oncornaviruses by caffeine. Biochem. Biophys. Res. Comm. 91: 239-246, 1979.

Reddy, E.P., Dunn, C.Y., and Aaronson, S.A.: Different lymphoid cell targets for transformation by replication competent Moloney and Rauscher leukemia viruses. Cell 19: 663-669, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05057-02-LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical and Immunologic Approaches Toward Identification of Sarcoma Coded Proteins.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Cremer	Senior Staff Fellow	LCMB	NCI
Other:	S.A. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	S. Varesio	Visiting Fellow	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	E. Canaani	Expert	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH	Carcinogenesis Intramural Program Laboratory of Cellular and Molecular Biology
SECTION	Molecular Biology Section
INSTITUTE AND LOCATION	Division of Cancer Cause and Prevention NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Replication-defective type-C sarcoma viruses arose as a result of recombination between replication-competent type-C retroviruses and host cellular sequences. In the Moloney murine sarcoma virus system (M-MSV) the incorporated cellular sequences have been shown to be involved in the sarcoma virus mediated transformation of fibroblasts in vitro. Current research is designed to identify and isolate the M-MSV coded messenger RNA(s) which are responsible for the process of cellular transformation. Messenger RNAs from nonproducer transformed cell lines are purified by molecular hybridization to specific fragments of M-MSV plasmid DNA bound to nitrocellulose filters. Sarcoma-specific RNA is eluted and translated in a reticulocyte cell-free system to identify the protein(s) product(s) which may be involved in fibroblast transformation. Sera from animals bearing M-MSV-induced tumors are tested for their ability to immunoprecipitate sarcoma coded proteins.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. To determine molecular mechanisms involved in the generation of different sarcoma virus isolates and variants.

2. To identify and characterize transformation specific protein(s) of mammalian sarcoma viruses.

3. To develop sensitive, reliable and accurate methods for the detection of sarcoma virus-related proteins in transformed cell systems and in normal cells and tissues.

4. To search for the expression of sarcoma virus-related proteins in naturally occurring tumors.

Major Findings:

Sarcoma-coded messenger RNAs from M-MSV nonproducer transformants were purified by molecular hybridization of polyadenylated cellular RNA to M-MSV sarcoma-specific cloned plasmid DNA. The sarcoma-specific mRNA(s) was eluted intact and was shown to be biologically active when translated in a reticulocyte cell-free translation system. Three sarcoma coded protein products, separated by gel electrophoresis, were identified as having molecular weights of 41,000, 38,000 and 24,000 daltons. The sarcoma viral RNAs of a number of different M-MSV isolates and natural deletion mutants were similarly prepared and translated, yielding proteins with similar but not identical molecular weights.

Significance to Biomedical Research and the Program of the Institute:

Sarcoma viruses contain cellular genetic information thought to code for proteins capable of transformation. The identification and characterization of sarcoma virus-coded transforming protein(s) thus provides a means of elucidating mechanisms involved in cellular transformation and the role of such proteins in naturally occurring tumors.

Proposed Course:

1. To continue to search for and identify translational products of mammalian sarcoma viruses involved in malignant transformation.

2. To develop assay systems for the identification and detection of biochemical processes involved in the establishment and maintenance of malignant transformation.

3. To search for and identify translational products of mammalian leukemia viruses involved in oncogenic transformation.

4. To search for and identify type-C viral proteins expressed in virus negative cell lines.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05058-02-LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mouse Endogenous Type-C Viruses: Gene Order and Evolutionary Relationships

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Merregaert	Visiting Fellow	LCMB	NCI
OTHER:	S.A. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project 1. Oligonucleotide fingerprinting analysis in conjunction with immunological analysis of several recombinant viruses generated both in vitro and in vivo from parental viruses with different oncogenic potential (between different Rauscher-MuLV ts and BALB:virus-2) enabled us to establish for the first time in the mouse leukemia system the exact order of the known leukemia virus genes. Project 2. In order to study the evolutionary pathways of mouse endogenous type-C viruses with respect to a common progenitor, we studied the distribution of different classes of inducible viruses among inbred strains of mice from diverse geographical areas.

PHS-6040
(Rev. 10-76)

Project Description

PROJECT 1

Objective:

To define the region of the murine leukemia viral genome critical to the neoplastic process.

Methods Employed:

A tissue culture protocol was devised for the generation and selection of recombinant viruses. Each recombinant was characterized to identify the origin of its translational products by means of appropriate competition radioimmunoassays. For each recombinant, T1-oligonucleotides derived from Rauscher-MuLV were positioned on its physical map.

Major Findings:

1. Gene order of murine leukemia viral genome as 5'-gag-pol-env-3'.

2. Physical map of Rauscher-MuLV.

3. Polyadenylation signal AAUAAA within terminal redundant sequences.

4. Concordance of RNA termini of viral recombinants.

Significance to Biomedical Research and the Program of the Institute:

Detailed biochemical and immunological characterization of viral recombinants in conjunction with *in vivo* experiments should allow us to determine which specific regions of the leukemia viral genome are required for leukemogenesis.

Proposed Course:

In vivo experiments to determine oncogenic properties of viral recombinants will be performed with the goal of determining which viral gene(s) are important for virus-induced leukemogenesis.

PROJECT 2

Objective:

Analysis of the distribution of endogenous type-C viruses in mouse strains from different geographical regions.

Methods Employed:

Iododeoxyuridine induction of fibroblastic primary cultures was followed by co-cultivation on sensitive assay cells. Clonal virus isolates were submitted to immunologic typing of their translational products, and their host range properties were studied.

Major Findings:

1. Class I and Class II viruses were shown to be genetically transmitted in a number of new inbred strains of mice and recombinants between them were detected.

2. The inducibility of prototype Class III virus from some inbred strains was established.

3. A new class of B-tropic-like viruses was characterized.

4. A mink-tropic-MuLV variant was isolated and characterized.

Proposed Course:

This project has been terminated.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05059-02-LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Development of Assay System for In Vitro Transformation by Murine Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Nagao	Visiting Fellow	LCMB	NCI
OTHER:	S.A. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH	Carcinogenesis Intramual Program Laboratory of Cellular and Molecular Biology
SECTION	Molecular Biology Section
INSTITUTE AND LOCATION	Division of Cancer Cause and Prevention NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using colony stimulating factors, culture techniques for long-term culture of normal hematopoietic cells have been established. Nontumorigenic bone marrow cells from NIH/Swiss, 129/J and C57BL/6 mice and spleen cells from NIH/Swiss, CBA and C57BL/6 mice have been maintained for over 12 months. Morphological and histochemical studies revealed that these cells are metachromatic cells capable of differentiation to basophilic granulocytes. These cells contain receptors for mouse IgE and histamine, which can be used as differentiation markers. Although these cells did not show colonies in spleens of lethally irradiated mice, colony and cluster formation was observed in methylcellulose medium, indicating that committed stem cells are maintained in suspension culture. These cell lines may be useful in studying the relationship between cell differentiation and viral oncogenesis.

Project Description

Objectives:

1. To establish a method of long-term culture of hematopoietic cells.

2. To examine optimal culture conditions to investigate differentiation of hematopoietic stem cells.

3. To examine the relationship between the differentiation stage of target cells and malignant transformation by murine leukemia viruses.

Methods Employed:

Different culture media are being tested to find the optimal conditions for long-term culture of hematopoietic target cells. Differentiation inducers and inhibitors are being examined to control the maturation of hematopoietic stem cells. Highly leukemogenic murine leukemia viruses have been isolated, and cloned viruses have been used to infect hematopoietic cells. Changes in differentiation markers of hematopoietic cells are being examined as well as changes in growth properties of these cells *in vitro* and *in vivo*.

Major Findings:

1. Hematopoietic cells from spleen and bone marrow have been maintained in suspension culture for periods of over 12 months. Culture methods for maintenance of committed stem cells have been established.

2. Morphologic and histochemical observation reveals that these cells have features of both basophylic myeloid cells and tissue mast cells. These cells contain receptors for IgE and histamine.

3. Cultures maintain the capacity for colony formation *in vitro*, indicating the persistence of progenitors (committed stem cells) of the more differentiated metachromatic cells.

4. The lack of tumorigenicity of these hematopoietic cells in syngeneic newborn and nude mice makes them potential targets for transformation by leukemia viruses in culture.

Significance to Biomedical Research and the Program of the Institute:

For understanding of viral leukemogenesis, culture methods for propagation of normal lymphoid and myeloid cells may be of great importance. Such techniques may make it possible to study the regulation of differentiation of normal hematopoietic cells and to test for *in vitro* transformation by oncogenic viruses.

Proposed Course:

1. Examine the stages of hematopoietic cell differentiation at which cells are susceptible to infection by a variety of murine leukemia viruses.

2. Analyze the effect of leukemia viruses and other agents on normal hematopoietic cell differentiation as well as their ability to cause malignant transformation.

3. Develop specific markers of differentiation of hematopoietic cells.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05060-02-LCMB

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of Oncogenic Expressions in Experimental Animal and Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any) S. Aaronson, LCMB, NCI; P. Arnstein, LCMB, NCI; D. Ablashi, LCMB, NCI; R. Adamson, LCHPH, NCI; D. Fish, FCRC; R. Gilden, FCRC; J. Cicmanec, Litton-Bionetics; M. Gardner & S. Rasheed, USC; J. Frankel, U. of Florida; S. Kalter & R. Heberling, S. W. Found. for Res. & Ed.; C. Graham, U. of Albany

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Viral Immunology Section

INSTITUTE AND LOCATION
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National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to (1) establish and define a cell culture transformation system for identification of genetically defective individuals and people exposed to environmental carcinogens; (2) identify and rescue human and primate sarcoma (src) information; and (3) develop and test vaccines and other preventive measures to prevent and control cell transformation and cancers in animal and primate systems and ultimately in humans.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. To develop a cell culture transformation system for identification of individuals at high risk for early cancer.

2. To search for and rescue human and primate sarcoma (src) information from primate and human cancers.

3. To develop a vaccine and other immunopreventive measures to prevent and control transformation and cancer in animals and ultimately in humans.

Methods Employed:

Biological methods include cell and virus cloning, transformation focus, cell aggregation, soft agar and genome rescue assays. Biochemical methods include RNA-dependent DNA polymerase, radio-immunoprecipitation assay and I^{125} protein A assay.

Major Findings:

1. High-titered ($>10^5$) Ki-MSV stock viruses were prepared for use as viral assays of relative risks (+) or (-) in cancer family syndromes such as ACR and Gardner's. Freshly harvested supernatant fluids of Ki-MSV transformed NRK cells were subjected to density gradient purification using zonal ultracentrifuges with RNA-free sucrose gradients, and the banded, purified virus was resuspended in Tris buffer (pH 7.0) at a concentration of 500 X.

Human skin cell lines (Risk + and Risk -) furnished and tested by Drs. Rasheed and Kopelovich were tested for focus formation by Ki-MSV. The results confirmed the genetic differential susceptibility of cultured human skin cells to transformation of Ki-MSV. Thus tests for identification of high risk individuals in families carrying autosomal dominant genes for cancer were confirmed in three laboratory test systems.

2. Increased transformation efficiency of Adeno 12/SV40 was observed in human skin cultures derived from high risk autosomal dominant cells. Transformation was evidenced by morphological alteration and presence of Adeno 12 and SV 40 "T" antigens by means of complement-fixation test. These transformed cells contained low levels of virus. However, they formed large cell aggregates and colonies in soft agar and then became permanent lines.

3. Human skin fibroblasts derived from ACR individuals are sensitive to a chemical carcinogen. An MNNG-treated human skin (PF) line showed morphological alteration and formed large cell aggregates above an agar overlay and formed colonies in soft agar. "Transformed cells" were resistant to rechallenge of MNNG (1 μg/ml) and showed a prolonged life span compared to untreated cells. However tumors were not produced in nude mice by subcutaneous inoculation. Attempts to produce tumors in nude mice by eye injection are in progress. Chemical carcinogens alone may not induce neoplastic transformation of fibroblasts from humans genetically predisposed to cancer. Neoplastic transformation of human skin cells by a chemical carcinogen apparently requires a multiple process.

4. Transformation of chimp skin cells by RNA and DNA tumor viruses. Chimp skin cells can be readily transformed *in vitro* by MSV(BaLV), and Ki-MSV transformed chimp skin cells were found to be virus producers, whereas RSV transformed chimp skin cells were found to be nonproducers (NP). Adeno 12/SV40 transformed chimp skin cells contained Adeno 12/SV40 "T" antigens and were found to be low virus producers. One NP Adeno 12/SV40 transformed chimp skin (WES) line was isolated. Tumors were produced when the NP cells were transplanted into nude mice. MSV(BaLV) transformed chimp skin cells produced tumors when the transformed cells were transplanted in nude mice. However, the tumors produced were murine tumors. Various clonal lines were established and characterization studies are in progress.

5. Induction of tumors in chimps by virus or chemical carcinogen. Attempts to induce tumors in chimps by inoculating MSV(BaLV), Adeno 12/SV40, and a chemical carcinogen (DENA) are in progress. MSV(BaLV) or Adeno 12/SV40 transformed chimp skin cells were also inoculated in chimps for tumor induction, but no tumors have been obtained to date.

6. Isolation of S^+L^- (sarcoma-positive, leukemia-negative) human osteosarcoma (HOS) clones induced by MSV(FeLV). Clones of S^+L^- HOS cells induced by MSV(FeLV) were isolated. S^+L^- HOS clones did not release infectious virus. S^+L^- HOS clones were tumorigenic in nude mice, whereas the nontransformed, parent HOS cells were nontumorigenic. However, S^+L^- HOS clones contained MSV in a rescuable form and expressed murine leukemia virus (MuLV) specific antigens such as the *gag* gene product (p15, p12, and p30) but lacked the *env* gene protein (gp 70). Nonproducer HOS cells isolated from transformed foci induced by Ki-MSV produced neither infectious virus nor MuLV antigens, but did contain a rescuable MSV genome. The NP cells produced tumors when transplanted subcutaneously into nude mice.

7. Inhibition of MNNG-induced transformation of canine cells *in vitro* by infection with mouse xenotropic type C virus. C57L mouse xenotropic type C virus infection inhibited N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced *in vitro* transformation of embryo cells from inbred beagles. Treatment with MNNG induced *in vitro* neoplastic transformation in uninfected

canine cells but not in C57L virus-infected canine cells; the C57L virus-infected canine cells not treated with MNNG also remained untransformed. In this dog cell system, preinfection with a C57L mouse xenotropic type C virus inhibited *in vitro* neoplastic transformation induced by MNNG.

Significance to Biomedical Research and the Program of the Institute:

Development of reproducible testing systems for identification of relative genetic susceptibilities to cancer is much needed in cancer diagnosis. Development of primate and human cell lines for rescue and identification of primate and human common tumor antigens will lead to eventual application to the development of protective vaccines against primate and human cancers.

Proposed Course:

1. To define further the Ki-MSV human skin transformation system and establish its validity and parameters as possible diagnostic indices for detection of hereditary cancer in man.

2. Examine further and confirm the increased transformation efficiency of Adeno 12/SV40 in human skin fibroblasts derived from genetically abnormal individuals.

3. Attempt to confirm and define an MNNG transformation system of human skin fibroblasts derived from ACR. Kopelovich *et al.* (1979) recently reported that tumor promoter alone (TPA) induces neoplastic transformation of fibroblasts from humans genetically predisposed to cancer. The role of TPA as well as nontransforming type C RNA virus in MNNG transformation of a human skin cell system will be studied in order to study cancer promotion *in vitro*.

4. To develop chimpanzee tumors transplantable in chimpanzees to serve as allogeneic vaccines and syngeneic challenge.

5. To rescue src expressions specific for primate and human tumors. The following efforts will be pursued.

A. Induction of tumors in chimps by tumor viruses or chemical carcinogens.

B. Induction of tumors in ATS-treated chimps by human tumor cells. Chimp tumors when confirmed by transplantation in nude mice will be transplanted into autochthonous chimp in order to amplify src sequences. Such tumors may be useful for isolation of a chimp sarcoma virus.

C. Isolation and characterization of variants from human and primate cells transformed by RNA tumor viruses or DNA tumor viruses.

D. Isolation and characterization of revertants from $S^{+}L^{-}$ human HOS cells which may provide a potentially important model for the understanding of the mechanism involved in cellular gene expression and tumorigenicity.

E. Seek cocultivation of chimp tumor cells that have been transplanted in a natural host one, two, or three times, based on rescue of rat src sequences.

6. Examine the protective effect against tumors of dogs and cats by infection with xenotropic type C viruses.

Publications:

Yang, Y.H., Rhim, J.S., Rasheed, S., Klement, V., and Roy-Burman, P.: Reversion of Kirsten sarcoma virus transformed human cells: Elimination of the sarcoma virus nucleotide sequences. J. Gen. Virol. 43: 447-451, 1979.

Cho, H.Y., and Rhim, J.S.: Cycloheximide-dependent reversion of human cells transformed by murine sarcoma virus and chemical carcinogen. Science 205: 691-693, 1979.

Rhim, J.S., Nelson-Rees, W.A., and Essex, M.: Transformation of feline embryo cells in culture by a chemical carcinogen. Int. J. Cancer 24: 336-340, 1979.

Rhim, J.S., and Arnstein, P.: Inhibition of chemical-induced carcinogenesis of canine cells in vitro by infection with mouse xenotropic type C virus. J. Nat. Cancer Inst. 64: 345-352, 1980.

Snyder, H.W. Jr., Essex, M., Shiski, A.H., Hardy, W.D. Jr., and Rhim, J.S.: Feline oncornavirus-associated cell membrane antigen. Cold Spring Harbor Symposium on Quantitative Biology. (In press.)

Rhim, J.S., Arnstein, P. and Huebner, R.J.: Chemical transformation of cultured skin fibroblasts from humans genetically predisposed to cancer. The 4th International Symposium on the Prevention and Detection of Cancer. London, 1980. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05061-02-LCMB

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Genome Organization of Abelson Murine Leukemia Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Srinivasan	NIH Visiting Fellow	LCMB	NCI
Other:	E. P. Reddy	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In an attempt to characterize the structural organization of Abelson murine leukemia virus, we have cloned the integrated proviral DNA of A-MuLV from EcoRI digests of transformed mink cell genomic DNA in bacteriophage λgt WES·λB. The proviral DNA containing phages were identified using Moloney MuLV cDNA and are being analyzed by restriction enzyme digestions, heteroduplex methods and infectivity.

PHS-6040
(Rev. 10-76)

Project Description

PROJECT 1.

Objectives:

To study the genome organization of Abelson murine leukemia virus.

Methods Employed:

Restriction enzyme digestions, RPC-5 chromatography, nucleic acid hybridization and molecular cloning techniques.

Major Findings:

DNA isolated from mink cells transformed by Abelson MuLV was cut with restriction enzyme EcoRI and the fragments were fractionated by RPC-5 chromatography. Proviral DNA containing EcoRI fragments were further enriched by preparative gel electrophoresis. These fragments were ligated to bacteriophage λgt WES·λB and packaged into phage particles in vitro. Hybrid phages were obtained using MuLV cDNA and are being analyzed by restriction enzyme digestions, heteroduplex methods and infectivity.

Significance to Biomedical Research and the Program of the Institute:

A-MuLV, isolated by passage of MuLV in steroid treated mouse, appears to have arisen in nature by a mechanism involving genetic recombination between MuLV and host cell nucleotide sequences. Fine structure analysis of A-MuLV may elucidate the relationship of its cell-derived nucleotide sequences to genetic information of the normal cell and its role in transformation.

Proposed Course:

A-MuLV unique region will be studied by DNA sequencing methods.

PROJECT 2.

Objectives:

To study the mechanism of action of caffeine.

Methods Employed:

Biochemical techniques involving column chromatography and assay of DNA polymerase activity using heat denatured, activated and synthetic DNA templates.

Major Findings:

Caffeine was found to inhibit repair enzyme DNA polymerase I from Escherichia coli. The inhibitory effect of caffeine was evident with the activated T_4, adenovirus 2 and calf thymus DNA and synthetic templates. This suggests that sensitizing effect by caffeine observed with different radiation and chemical mutagens may be due to this inhibitory effect. DNA polymerase from Micrococcus lysodeikticus (which resembles DNA polymerase I) is also inhibited. Kinetic experiments using different template concentrations indicate that the target of caffeine may be template itself.

Significance to Biomedical Research and the Program of the Institute:

Tumor cells have the capability to repair damage inflicted on DNA by physical and chemical therapeutic agents. In light of this, use of repair inhibitors in combination with therapeutic agents would be highly desirable for the efficacy of the treatment of tumors. The possibility of caffeine being used in combination with therapeutic alkylating agents would be worth attempting on the basis of this finding.

Proposed Course:

Further studies towards understanding whether caffeine effect is specific only to DNA repair enzyme will be attempted.

Publications:

Srinivasan, A., Reddy, P., Sarma, P.S., and Schultz, R.A.: Inhibition of RNA-dependent DNA polymerase activity of oncornaviruses by caffeine. Biochem. Biophys. Res. Comm. 91: 239-246, 1979.

Balachandran, R., Srinivasan, A., and Reddy, P.: Caffeine inhibits repair enzyme DNA polymerase I from Escherichia coli. Studies in vitro. Carcinogenesis. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05062-02-LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Virus-Specific Messenger RNAs in M-MSV Infected Nonproducer Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Eva-Varesio	Visiting Fellow	LCMB	NCI
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	M. Barbacid	Expert	LCMB	NCI
	E. Canaani	Expert	LCMB	NCI
	K. Robbins	Expert	LCMB	NCI
	S.R. Tronick	Microbiologist	LCMB	NCI
	K. Cremer	Staff Fellow	LCMB	NCI
	S. Devare	Visiting Associate	LCMB	NCI

COOPERATING UNITS (if any)

None

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Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

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Molecular Biology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Moloney murine sarcoma virus (M-MSV) is a replication-defective retrovirus that transforms fibroblasts in vitro. It has been demonstrated that M-MSV arose by recombination between the replication-competent Moloney-MuLV and a specific subset of cellular sequences. These sequences present in M-MSV are known to be involved in cellular transformation mediated by the virus. The purpose of this work is to identify the messenger RNAs of M-MSV involved in the cellular transformation. The study will be focused on those RNAs coded for by different clones of M-MSV deletion mutants. Total cellular RNA from nonproducer transformants will be analyzed by the "Northern gel technique" and the M-MSV specific RNA detected by molecular hybridization. The sequence organization of these viral RNAs will be characterized by utilizing specific [^{32}P]-labeled M-MSV DNA probes corresponding to specific regions of the viral genome. Eventually the putative translational products of such an RNA molecule will be analyzed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

Detection and characterization of the transcriptional products of the M-MSV src gene and their involvement in malignant transformation.

Major Findings:

1. Techniques have been developed to isolate and purify from cells total and messenger RNAs, to covalently couple the RNAs to the diozobenzyloxymethyl paper and to detect them by hybridization to [^{32}P]-labeled M-MSV DNA resuspending either full length or subgenomic fragments of the viral genome.

2. Genomic-length as well as subgenomic polyadenylated RNAs have been detected in cells transformed with different M-MSV deletion mutants. These subgenomic RNAs contained the M-MSV src specific sequences as detected by hybridization to ^{32}P-labeled M-MSV src-specific DNA fragments.

Significance to Biomedical Research and the Program of the Institute:

Since the RNA sarcoma viruses have been shown to cause cancer in animals and to transform fibroblasts in cell culture, identification and characterization of the M-MSV src gene messenger RNA and its translational product(s) can help in understanding the mechanisms of virus-mediated cellular transformation.

Proposed Course:

1. Enhance the resolution of the techniques used to isolate the mRNAs for the purpose of identifying all possible M-MSV transcripts.

2. Prepare M-MSV ^{32}P-labeled DNA fragments as molecular probes in order to determine the complexity and sequence organization of each of these RNAs.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05063-02-LCMB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on Epstein-Barr Virus, Herpesvirus Saimiri, and Herpesvirus Ateles

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	J. Rice	Chief, Perinatal Carcinogenesis Section	EXP	NCI
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COOPERATING UNITS (if any) R. Krueger, Pathology Inst., Univ. of Cologne, West Germany; M. Nonoyama, Life Sciences Inc., St. Petersburg, Fla.; G. Pearson, Mayo Clinic, Rochester, Minn.; A. Bouguermouh, Inst. Pasteur of Algerie, Algeria; U. Prasad, Univ. of Malaya, Malaysia

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Increasing immuno-virological and biochemical evidence supports the concept that the Epstein-Barr virus (EBV) is the major contributing factor in the multifactorial etiology of nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma. The other contributing factors, such as chemicals, immunosuppressants, and genetic predisposition, interacting with the EBV, undoubtedly play a profoundly important role in induction of these cancers. Moreover, Herpesvirus saimiri (HVS) of the squirrel monkey, and Herpesvirus ateles (HVA) of the spider monkey are prevalent and widely distributed in these monkeys and are known to induce tumors in a variety of nonhuman primates as a result of inoculation of virus or by horizontal spread of virus. For all of these reasons, the cellular and humoral responses of animals bearing these virus-induced tumors are important models for application to diagnosis, prognosis, evaluation of antiviral anticancer drugs and antitumor vaccine preparations in relation to EBV virus activity in humans.

Project Description

Objectives:

A. To study the herpesviruses associated with neoplasia in humans (NPC and BL) in nonhuman primates, inbred rabbits and athymic nude mice; and to examine the interaction of tumor promoting agents (i.e., TPA) weak carcinogens and/or other cancer-predisposing factors, such as immunosuppressants with the herpesviruses *in vitro* and *in vivo*.

B. To initiate and participate in an international collaborative research program on herpesviruses of primates which includes my laboratory and other national and international institutions and organizations.

Methods Employed:

A. Primary cell cultures and continuous cell lines of human and animal origin were used for virus isolation, biological and biochemical assays, and for *in vivo* experimentation. Standard biochemical and immunological procedures were applied. Human subjects were involved in the research projects by obtaining sera from patients with various cancers of interest, healthy donors, and individuals working in the laboratory with oncogenic herpesviruses.

The interaction of tumor promoting agents, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and saccharin, as well as carcinogens such as MMS and MNNG were examined by employing such chemicals in combination with herpesviruses to determine their effects (i.e., enhancement) on infectious virus and viral antigens, transformation, and *in vivo* effects in nonhuman primates and other laboratory animals.

Lymphoblastoid cell lines established from Burkitt's lymphomas (American and African) were tested in nude mice for tumorigenicity and viral genome regulation.

Major Findings:

A. The interaction of TPA, a known tumor promoter, was found to enhance EBV transformation of mononuclear cells from adult white-lipped and common marmosets. Since we found that TPA was mitogenic to such cells and had a synergistic effect with phytohemagglutinin (PHA), mononuclear cells were treated with PHA-P prior to infection with EBV and then allowed to incubate in the presence of TPA which resulted in 4 out of 4 transformations, whereas only 1 out of 4 cultures treated with virus alone and 3 out of 4 treated with virus plus TPA resulted in clones of transformed cells.

TPA interaction with human-simian cells shedding nontransforming (P3HR1) and transforming (B95-8) EBV showed significant stimulation of early antigen (EA) and virus capsid antigen (VCA), as well as 1.0-1.5 logs more

virus. EBV cell lines of recent (<20 passages) origin gave significantly lower EA and VCA, suggesting that such cell-virus interactions may be influenced not only by the dose of TPA but also by in vitro passage of cells. TPA and cell-virus interaction was also influenced by cell density and cell viability.

Continuous treatment of EBV nonproducer cells with TPA induced some percentage of EA-producing cells without any evident toxicity. This finding provides justification for such treatment of cells established from NPC biopsies in efforts to transform cultures and establish permanent cell lines.

Human peripheral mononuclear cells stimulated by TPA released a soluble lymphocyte factor (lymphokine) which was found to be a protein. The release of this factor was inhibited by mitomycin C treatment.

Retinoic acid (vitamin A) inhibited TPA-induction of EA in EBV nonproducer, genome carrying cells, but failed to inhibit mitogenic activity of TPA in human lymphoblastoid cells, suggesting two independent mechanisms of action. Moreover, retinoic acid inhibition of virus induction by TPA with no diminution of antigen synthesis after superinfection of nonproducer cells by nontransforming EBV suggests that the TPA may be activating the genome indirectly by a common mediator which is blocked by retinoic acid. The blocking of EA further suggests an indirect effect on EBV-DNA polymerase.

The interaction of TPA with transforming EBV was tested further in common marmosets, where EBV was known to be immunogenic but did not induce tumors. Inoculation of EBV into the bone marrow, followed by intraperitoneal TPA treatment resulted in diffuse malignant lymphoma. The animal was also found to be positive for EA (1:40) and VCA (1:160) and a depressed cell-mediated immune (CMI) reponse to PHA-P. EBNA-positive cells and multiple copies of EBV-DNA were found in the liver and mesenteric lymph nodes.

The animal which had received TPA alone demonstrated a depletion in blood platelets and died with no signs of tumor. The animal receiving EBV alone demonstrated a low EBV antibody titer, and died without any tumors as well. Thus it appeared that the induction of malignant lymphomas required both the initiator EBV and promoter (TPA).

Subtoxic (0.5 - 2.0 ng/ml) levels of a direct alkylating agent, n-methyl-n'-nitrosoguanidine (MNNG), were found to enhance HVS titers in owl monkey kidney cells from 100-1000 fold when cells were treated in their stationary phase of growth 24-48 hours prior to HVS infection. Post-treatment with MNNG was also effective, but not to the same extent. MNNG treatment also significantly enhanced HVS-EA and late antigen producing cells. However, actively growing cells treated with MNNG did not give any significant increase in HVS titers. Moreover, both pre and post viral treatment was dose and time dependent.

Pretreatment of human cord blood mononuclear cells with subtoxic doses of MNNG (0.5 and 0.1 ng/ml) for 48 hours resulted in earlier transformation by EBV and required 1.0 - 1.5 logs less virus. MNNG failed to induce EA in nonproducer genome carrying EBV and HVS cells, suggesting that its mechanism of action is different from that of TPA.

The partially purified prototype strain of HVS, administered IV and IM in 4 inbred rabbits (oryctolagus cuniculus) less than a year of age induced poorly differentiated lymphoma in 33-59 days. Humoral and cellular responses during tumorigenesis were comparable to those induced by HVS in nonhuman primates. Thus the inbred rabbit strain III/J appears to be a suitable model for immunovirological, chemotherapeutic and preventive vaccine studies of oncogenic herpesviruses-induced cancer. Inbred rabbits are less expensive than primates and are easily bred in adequate numbers.

The effectiveness of interferon derived from human leukocytes (LI), lymphoblasts (LYI), fibroblasts (FI) and the polynucleotide interferon inducers Poly (I): Poly (C) and Poly ICLC was tested and compared against HVS and EBV. Applied after virus adsorption, all preparations were demonstrated to be active against HVS and EBV, although different dosages of each were required to achieve 50% inhibition of viral effects. Transformation of human umbilical cord blood lymphocytes by transforming EBV in low multiplicity of infection was prevented by pretreatment of the cells with high doses of LI and LYI. The most effective inhibitor of HVS and EBV was LI, and least effective was FI. The inhibitory effects of Poly (I): Poly (C) and Poly ICLC on HVS and EBV was dependent on their ability to induce interferon which varied with individual cell systems. Owl monkey kidney cells which were responsive to the inducers required nanogram levels of polynucleotides to develop resistance to HVS-induced CPE. Transformation by EBV was also prevented by Poly (I): Poly (C) only when the multiplicity of infection was low. Spontaneous expression of EBV-associated nuclear antigen and VCA was not affected by interferon or polynucleotides. Thus it is possible that differences in vitro may accurately reflect the sensitivity of oncogenic herpesviruses to different types of interferon in vivo.

B. (1) An international study on NPC was initiated between the University of Köln, West Germany; the Institute Pasteur, Algeria; University of Malaysia; Mayo Clinic, Rochester, Minnesota; and Life Sciences, Florida, to investigate the humoral and cell-mediated immunities to EBV. In this collaborative study, we compared EBV serology in relation to histopathology, clinical stages, and racial groups to develop profiles which would help in diagnosis and prognosis. All NPC cases, regardless of sex, race and country of origin were found to have significantly higher levels of antibodies to EBV antigens (VCA, EA, EBNA and complement fixing antigens) when compared to controls. Those with elevated EBV-VCA IgA antibody were found to have a poor prognosis in the course of the one-year's study. This antibody either remained unchanged or increased with tumor load, and decreased when the patient responded to therapy. Antibody-dependent

cytotoxicity (ADCC) levels were found to be higher in those patients which had lower IgA antibodies, perhaps reflecting a blocking effect. The ADCC titers increased in two patients from West Germany coincident with a drop in IgA titers from 1:160 to 1:10. The imprints from biopsies from Algeria and Malaysia demonstrated the presence of EBNA in the epithelial cells in all of the undifferentiated carcinomas. All of the latter cases also contained IgA antibody. Some poorly differentiated carcinomas were also EBNA-positive. However, well and moderately differentiated carcinomas lacked EBNA although IgA antibody was detected in sera from these patients. In limited EBV DNA-DNA hybridization studies on two biopsies, 7-9 genomes/cell were detected. The median age of more than 90% of German, American and Malaysian NPC cases was 45 - 50 years, with a 3:1 ratio of males to females. In 37 Algerian cases, two age peaks were seen: one $\leq$20 years and the second at age 40 and above. These findings are inconsistent with earlier reports of NPC age distribution in Tunisia where the ratio of males to females at $\leq$20 years was 1:1 and the same (3:1) in those over 40 years of age.

(2) Under the WHO/FAO program on comparative virology, my laboratory prepared the high titered PPLO-free, purified prototype HVS which serves as the reference virus. This was deposited with the WHO Collaborating Center for Reference and Reagents in Simian Viruses, San Antonio, Texas.

Significance to Biomedical Research and Program of the Institute:

The findings under A and B(1) on EBV, HVS, and HVA relate to clinical and laboratory studies which confirm and extend previous studies and initiate new approaches in biochemical, immunovirological, pathological and virus-chemical interactions designed to define the interactions and immunological parameters of the herpesviruses associated with neoplastic disease. It is hoped all of the information developed will help provide valuable clinical tools for diagnosis, prognosis, and better control of herpesvirus-associated neoplasms. The cell-virus-chemical interactions support a multifactoral etiology of tumors implicating the herpesviruses etiologically. This work also suggests that host and other factors (tumor promoters, and weak carcinogens) do affect the outcome of herpesvirus infection, cell transformation and possible tumor induction. Some of the *in vitro* findings may also be applicable t` *in vivo* interactions contributing to oncogenesis.

The interaction of tumor promoters with oncogenic virus cell systems may prove a valuable tool in screening the effect of chemicals which do not react in the Ames test. To my knowledge, agents like TPA and saccharin do not respond in the Ames test. Moreover, primate virus cell systems have greater relevance for humans than rodent or bacterial systems.

Proposed Course:

The project will be continued. More emphasis will be placed on in vivo interactions and immunoprevention approaches of herpesvirus-induced tumors in primates, rodents and nude mice.

1. Since the most significant EBV antibodies in NPC appeared to be IgA and EA-D which relate to the tumor progression and regression, follow up serum samples will be taken from surviving cases during and after treatment, during remissions and at the time of reappearance of tumor in order to define what relationship IgA and EA antibodies have to prognosis.

2. Attempt will be made to propagate tumor cells (epithelial) and isolate virus from biopsies positive for EBV-DNA in order to find EBV strain differences.

3. Study the effect of EBV antibodies in NPC patients to determine which antibodies increase with tumor burden, or decrease or disappear in the absence of detectable tumor, and how early such antibodies reappear.

4. Family members of NPC patients and certain HLA type individuals (A2B Sin2, AWB17, A9B18) of Chinese or Malaysian origins will be followed for EBV IgA in order to determine whether they are at unusual risk for NPC.

5. Continue to review and correlate NPC histological classifications in relation to EBV experience.

Publications:

1. Ablashi, D.V., Gerber, P., and Easton, J.M.: Oncogenic herpesviruses of nonhuman primates. Comp. Immunol. Microbiol. and Inf. Dis. 2: 229-241, 1979.

2. Ablashi, D.V., Bengali, Z.H., Daniel, M., Eichelberger, M.A., Sundar, K.S., and Levine, P.H.: In vitro interaction between oncogenic herpesviruses of primates and tumor promoting agent. Bibliotheca Haematologia, Comparative Leukemia Research, 1979. New York, S. Karger (In press).

3. Sundar, K.S., Ablashi, D.V., Bengali, Z.H., Levine, P.H., and Nonoyama, M.: Mitogenic effects of 12-O-tetradecanoyl-phorbol-13-acetate on nonhuman primate mononuclear cells and in vitro interaction with Epstein-Barr virus transformation. Arch. of Virol. (In press).

4. Kalter, S.S., Ablashi, D., Espana, C., Heberling, R.L., Hull, R.N., Lennette, E.H., Malherbe, H.H., McConnell, S., and Yohn, D.: Simian virus nomenclature. Intervirology, 1980 (In press).

5. Ablashi, D.V., Bengali, Z.H., Eichelberger, M.A., Sundar, K.S., Armstrong, G.R., Daniel, M., and Levine, P.H.: Increased infectivity of oncogenic herpesviruses of primates with tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate. Proc. Soc. Exptl. Biol. Med. (In press).

6. Ablashi, D.V., deThe, G., and Ito, Y.: International symposium on etiology and control of nasopharyngeal carcinoma. In 1980 Year Book of Cancer. The Univ. of Texas Cancer Center (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05073-02 LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Modulation of Cell Surface Receptors for the Major Envelope Glycoprotein of Murine Leukemia Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Twardzik	Biochemist	LCMB NCI
Other:	A. K. Fowler	Head, Bio-molecular Oncology Section	LCMB NCI
	P. T. Allen	Microbiologist	LCMB NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Bio-molecular Oncology Section

INSTITUTE AND LOCATION
DCCP, NCI, NIH
Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The modulation of murine thymocyte surface receptors for the 71,000 dalton major envelope glycoprotein, gp71, of Rauscher murine leukemia virus has been examined utilizing a highly specific [^{125}I] radiolabeled gp71 binding assay. Cell surface receptors for ecotropic murine leukemia virus respond similarly to ligand-induced topographical modulation as do other cell surface receptors indicating that their anchorage and movement in the cell membrane is mediated via microfilament and microtubule cytoskeletal structures.

A reduction in the affinity constant, K_a, and number of available receptor sites on thymocytes for gp71 by adenosine triphosphate has been demonstrated. This effect is specific in that no modulation is seen with the other ribonucleotides, deoxynucleotides or cAMP, ADP or AMP. The inclusion of an ATP generating system in the receptor assay reduced the amount of ATP needed to reduce gp71 binding. Non-hydrolyzable analogs of ATP, i.e. App(NH)p, also modulated receptor activity suggesting an allosteric interaction of ATP with the gp71 receptor complex.

Project Description

Objectives:

The objectives of this project are to (a) characterize the cell surface receptor for the 71,000 dalton major envelope glycoprotein of ecotropic murine leukemia virus; (b) identify the active site on the gp71 molecule involved in cell surface receptor recognition; and (c) determine the involvement of cytoskeletal structural elements in the mobility and distribution of gp71 receptors in the plasma membrane and the modulation of receptor affinity by allosteric transducers.

Methods Employed:

The major 71,000 dalton envelope glycoprotein of R-MuLV was purified utilizing non-denaturing conditions and radioiodinated using the chloramine T procedure to specific activities of approximately 2.5×10^4 cpm [^{125}I]/ng of purified viral glycoprotein. This probe was used to develop a specific binding assay system (Fowler et al., J. Virol.) to quantitate the number of available gp71 receptor sites on susceptible target cells. Thymic cells (1×10^6-10^7) from BALB/c mice were incubated at 37°C in the presence of approximately 3-5 ng of [^{125}I]-gp71 in RPMI 1640 media containing 1% bovine serum albumin. After incubation, cells were centrifuged at 400 xg for 5 minutes at 4°C, washed 3 x with RPMI-BSA, and the radioactivity associated with the cell pellet determined. Non-specific binding to the reaction tube was determined in control cultures containing no cells.

Modulation of available cell surface receptor for gp71 was examined by either pretreatment of cells with ribonucleotides prior to determining bound [^{125}I] gp71 or by the simultaneous addition of probe and ribonucleotide, i.e. ATP. Equilibrium affinity constants, K_a's, and number of receptor sites per cell was determined as previously described (Stachard) utilizing a computer directed Stachard analysis program developed by Robard at the NIH. Constants were determined by computerized best fit linear regression analysis.

The influence of local anesthetics on the distribution of gp71-receptor complexes on normal thymocytes and endogenous gp71 in the membranes of C3HMT1820 thymoma cells persistently infected with R-MuLV was examined by fluorescent antibody analysis using anti-gp71-induced capping. Monospecific goat anti-gp71 (1:200 dilution) was incubated with 1×10^6 C3HMT1820 thymoma cells for 60 minutes at 4°C. After washing, cells were incubated with pig anti-goat IgG labeled with fluorescein isothiocyanate diluted 1:10 in PBS. Local anesthetics were then added, temperature raised to 37°C and cultures reincubated for 1.5 hrs. Cells were fixed with 0.25% glutaraldehyde and examined for the distribution of the fluorescence with a Zeiss ultraphot microscope. The percentage of capped, patched or diffusely stained cells was determined by counting at least 200 glutaraldehyde fixed lymphocytes.

Native [^{125}I]-gp71 was cleaved with a variety of proteolytic enzymes either with or without prior removal of select carbohydrate groups and the apparent molecular weight of the generated fragments was determined utilizing SDS-polyacrylamide gel electrophoresis. Cleavage products were then resolved into individual components by lectin affinity columns followed by gel permeation chromatography. Fragments of gp71 were then tested in a binding assay for cellular binding characteristics and for competition by unlabeled parent gp71. The relationship of these fragments to gp71 was also determined utilizing two-dimensional tryptic mapping procedures as previously described.

Major Findings:

Susceptible murine thymocytes efficiently bind [^{125}I]-gp71 from R-MuLV (1.5-2.5 ng/10^6 cells). The observed binding is linear for about 30 minutes at 37°C and undegraded 71,000 daltons glycoprotein can be recovered from the cell membrane. The binding of [^{125}I]-gp71 to thymocytes is blocked by monospecific antibody to gp71 and is competitively inhibited by unlabeled gp71 (50% competition with 2-3 ng/10^6 cells). Pretreatment of cells with 0.5 mM dibucaine reduced gp71 binding by 90%; all other local anesthetics reduced gp71 binding but only at 10-fold higher concentrations. This observed reduction in the binding of gp71 to anesthetic treated thymocytes was abrogated by supplementing anesthetic treated cultures with calcium.

The antibody-induced capping of the gp71 receptor complex was eliminated by pretreatment of cells with 5 mM lidocaine. Although 75% of the fluorescing cells demonstrated caps in buffer controls, none of the anesthetic treated cells exhibited capping. A similar effect was observed in the antibody-induced redistribution of endogenous cell membrane gp71 in C3HMT1820 thymoma cells. Whereas approximately 60% of the fluorescing cells formed caps in control buffer, only 10% formed caps in 5 mM lidocaine. A depression in antibody-induced patching of endogenous gp71 was also seen in anesthetic treated cells.

Limited digestion of gp71 with proteolytic enzymes covalently bound to sepharose beads generated fragments of gp71 with molecular weights of approximately 52,000, 30,000 and 15,000. The 30,000 dalton fragment binds to Con-A sepharose and shares many of the tryptic peptides with the parent gp71 molecule. This fragment efficiently binds to murine thymocytes, and the binding is competitively inhibited by unlabeled native gp71. This 30 kd fragment of gp71 also exhibits different divalent cation requirements when compared to the native molecule.

Significance to Biomedical Research and the Program of the Institute:

Studies on the molecular biology of cell surface receptors for horizontally transmitted virus are of obvious value. Of more importance, however, are those cellular mechanisms mediated by the interaction of endogenous envelope gene products gp71 and homologous receptor sites. For example, evidence is

accumulating in the murine system which suggests that leukemogenic transformation may be mediated by the interaction of gp71 and homologous receptors and the subsequent stimulation of mitogenesis. Studies on gp71-gp71 receptor interaction in the mouse may be a valuable model system with application to studies on human leukemia in the event that a similar mechanism of leukemogenesis can be established.

Proposed Course:

Experiments to further characterize and identify the cell surface receptor for gp71 will continue as will examination of those physiological parameters which modulate receptor availability and function. Efforts will be directed at further cleaving the 30 kd gp71 fragment into a polypeptide which maintains receptor recognition and is small enough to permit sequencing of the active receptor region of the gp71 molecule.

Publications:

Twardzik, D.R., Fowler, A.K., Weislow, O.S., Hegamyer, G.A., and Hellman, A.: Cell surface binding proteins for the major envelope glycoprotein of murine leukemia virus. Proc. Exp. Biol. Med. 162: 304-309, 1979.

Fowler, A. K., Reed, C.D., Riggs, C.W., Twardzik, D.R., Weislow, O.S., and Hellman, A.: Depression of Rauscher leukemia virus envelope glycoprotein gp71 binding by lymphoid cells during leukemogenesis in mice. Infect. Immun. 24: 647-655, 1979.

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Hellman, A., Weislow, O. S., Twardzik, D.R., and Fowler, A.K.: Type C retrovirus activation and possible functions in the normal and tumor bearing host. Cancer Res. 39: 2902-2907, 1979.

Twardzik, D.R., Reed, C.D., Weislow, O.S., and Fowler, A.K.: The effect of local anesthetics on cell surface receptors for the major envelope glycoprotein of murine leukemia virus. Int. J. Immunopharmacol. (in press).

Twardzik, D.R., Ranchalis, J.E., and Fowler, A.K.: Interaction of murine thymocyte histocompatibility antigens with envelope glycoproteins of Rauscher murine leukemia virus. In Lapin, B. (Ed.): Proceedings of the IXth International Symposium for Comparative Research on Leukemia and Related Diseases. U.S.S.R., Russia Publishing Firm, (in press).

Twardzik, D.R., Ranchalis, J.E., and Fowler, A.K.: Affinity of murine leukemia virus envelope proteins for H-2 gene products. In IXth International Symposium for Comparative Research on Leukemia and Related Diseases, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05110-01-LCMB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHERS:	S.A. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI
	S.R. Tronick	Microbiologist	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	E. Canaani	Expert	LCMB	NCI
	K. Cremer	Senior Staff Fellow	LCMB	NCI
	A. Varesio	Visiting Fellow	LCMB	NCI
	K. Robbins	Expert	LCMB	NCI
	A. Srinivasan	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

S. Devare, LCMB, NCI; P. Reddy, LCMB, NCI

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION ~~Division of Cancer Cause and Prevention~~
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this study is to biochemically characterize retroviruses in order to understand the mechanisms by which these viruses induce cancer in their natural hosts. The role of these viruses in the etiology of human cancers is also under study. Studies currently in progress are the following: 1) biochemical characterization of replication-defective mammalian transforming viruses; 2) search for the presence of retroviral genes and gene products in human tumors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. To biochemically characterize mammalian transforming retroviruses.

2. To determine the mechanisms of oncogenesis by retroviruses in their natural hosts.

3. To determine whether or not retroviruses play a role in the etiology of cancers of higher primates, including humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of tumor viruses and their proteins; molecular hybridization techniques using single-strand specific nuclease and hydroxyapatite chromatography to detect and characterize viral genomes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of cloned DNAs by restriction endonuclease mapping, nucleotide sequencing, and electron microscopy.

Major Findings:

Unintegrated 5.8 kbp closed-circular DNA of Moloney murine sarcoma virus (MSV) strain 124 was isolated from acutely infected cells. This DNA was cleaved to the genome-length linear form by the restriction endonuclease Hind III and ligated to Hind III-cleaved bacteriophage Charon 21A DNA. The recombinant DNA was packaged in vitro into phage particles and propagated in E. coli DP50SupF. Phages containing MSV-specific nucleotide sequences were identified by using molecular hybridization, and their DNAs were subjected to restriction enzyme and heteroduplex analyses. MSV-specific inserts of 6.4, 5.8, 5.2, 4.0 and 2.0 kilobase pairs (kbp) were observed. Several plaque-purified phages were each found to have different sized inserts (for example, 6.4, 5.8, and 5.2 kbp) and, in some instances, phages containing the smallest inserts were observed to accumulate following growth in E. coli. The 6.4, 5.8, and 5.2 kbp forms were found to differ in their degree of representation of 600 bp direct repeats (present at the termini of genomic MSV DNA). The 4.0 and 2.0 kbp inserts were generated from the larger species during propagation of the phage in bacteria and arose by deletion of a continuous segment of DNA starting in each case at about 700 bp from the 5' end of the cloned MSV molecule. Each DNA form was found to induce morphological transformation of cultured mouse cells. However, transforming virus could only be rescued from cells transfected by the 6.4, 5.8, and 5.2 kbp forms. Hind III cleaves the MSV genome within sequences necessary for transformation, but transformation was obtained with cloned MSV DNA that had not been ligated to the circular form. This suggests that ligation of such molecules occurred within the mouse cell, thus generating

biologically active molecules. MSV-124 unintegrated DNA was also cloned in Charon 16A by cutting with Xho I which cleaves in the helper-specific sequences of MSV-124. The MSV-specific DNA (5.8 kbp) cloned in Charon 16A was 10 - 100 times more transfectious than the Hind III cloned molecules.

The cloned MSV DNA was used to prepare probes specific for the helper and sarcoma virus-specific (src) sequences of MSV-124. These probes were then utilized to study the arrangement of helper and src-related sequences in normal mouse DNA. EcoRI, for which MSV DNA is not a substrate, was used to cut high molecular weight DNA isolated from several inbred mouse strains. The DNAs were subjected to gel electrophoresis, Southern blotting, and hybridization with each probe. The helper specific prove hybridized with many DNA fragments. In contrast, a single 14 kbp fragment, that did not coelectrophorese with any of the helper-specific bands, was detected with the src-specific probe. Thus, the src-specific sequences of MSV are derived from the normal mouse genome and are present in a configuration analogous to that of a cellular gene. Furthermore, the arrangement of these sequences is conserved in the DNA of several inbred mouse strains.

MSV DNA cloned in λ was subcloned in a plasmid vector (pBR322) to provide larger quantities of DNA for purposes of isolating MSV mRNAs by affinity chromatography and also for providing more readily probes specific for various regions of the MSV genome (i.e, helper, src, terminal redundancies). In the course of these experiments a sequence present in the src region of MSV was found to function as a promoter for the tetracycline resistance gene of pBR322. Nucleotide sequencing of this region identified a sequence very similar to classic procaryotic promoters. The location of this sequence on the MSV genome suggests a unique mechanism for transformation by MSV.

The unintegrated 8.8 kbp closed-circular DNA of simian sarcoma associated virus (SSAV) has been isolated from newly infected cells. This DNA was cleaved once by the restriction enzyme EcoRI, then cloned in the phage vector Charon 16A. The SSAV cloned DNA is currently being characterized. Nick translated probes made from cloned SSAV DNA are being used to identify for subsequent cloning the integrated simian sarcoma viral DNA from cells nonproductively transformed by this virus.

Significance to Biomedical Research and the Program of the Institute:

The availability of highly sensitive and specific biochemical and immunological probes for retroviral gene products has become increasingly of value in order to both demonstrate the possible etiologic involvement of these viruses in human cancers and to study the role that retroviral gene products may play in normal cellular functions.

Proposed Course:

A detailed analysis of the regions of the MSV genome required for transformation, integration, and rescue by helper virus is now under way by using various restriction enzyme-generated fragments of cloned MSV DNA.

The integrated form of MSV-124 and the MSV-related sequences in normal mouse cells are being cloned in order to gain an understanding of the mechanism of transformation by MSV and also to determine the role in oncogenesis of src-related sequences in normal mouse cellular DNA.

Recombinant DNA techniques are being applied and further developed to study other mammalian transforming viruses and cellular genes involved in transformation in species other than the mouse.

Publications:

Tronick, S.R., Robbins, K.C., Canaani, E., Devare, S.G., Andersen, P.R., and Aaronson, S.A.: Molecular cloning of Moloney murine sarcoma virus: Arrangement of virus-related sequences within the normal mouse genome. Proc. Natl. Acad. Sci. USA 76: 6314-6318, 1979.

Canaani, E., Tronick, S., Robbins, K., Andersen, P., and Aaronson, S.A.: Cellular origin of the transforming gene of Moloney murine sarcoma virus. Cold spring Harbor Symposium on Quantitative Biology, 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05111-01-LCMB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Isolation and characterization of the feline sarcoma virus (McDonough strain) genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Salzberg	Visiting Scientist	LCMB	NCI
OTHER:	S.A. Aaronson	Chief, Laboratory of Cellular and Molecular Biology	LCMB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH	Carcinogenesis Intramural Program Laboratory of Cellular and Molecular Biology
SECTION	Molecular Biology Section
INSTITUTE AND LOCATION	Division of Cancer Cause and Prevention National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to characterize the transforming gene of the McDonough (SM) strain of FeSV, two approaches were undertaken to isolate the SM-FeSV viral DNA. Unintegrated viral DNA was obtained from newly infected cells and analyzed by agarose gel electrophoresis, blotting, and molecular hybridization with appropriate cDNA probes. Major linear forms of 9.5 and 6.5 kbp, respectively, presumably represented helper and sarcoma viral DNA. A preliminary restriction endonuclease map of the unintegrated form of SM-FeSV DNA has been obtained. Restriction endonuclease and Southern blotting techniques have also been utilized to identify viral DNA fragments in the DNAs of SM-FeSV transformed NRK and NIH 3T3 cells. Appropriate sized fragments for molecular cloning have been identified. Following molecular cloning of the SM-FeSV genome, a more detailed restriction endonuclease map will be obtained and the transforming region of the genome will be mapped by analysis of transfection by subgenomic DNA fragments.

PHS-6040
(Rev. 10-76)

Project Number Z01-CP-05111-01-LCMB

Project Description

Objective:

Isolate and characterize the genome of SM-FeSV.

Methods Employed:

Our approach to the isolation and characterization of the genome of SM-FeSV was to use exogenously infected cells for the isolation of proviral DNA in its unintegrated form, and viral transformed nonproducer cells for the isolation of viral DNA in its integrated form. We have established a cloned line of NIH/3T3 mouse fibroblasts transformed by SM-FeSV. This cell line was generated by infecting NIH/3T3 cells with end point dilution of SM-FeSV carrying as its helper P. cynocephalus leukemia virus. The latter does not replicate in mouse cells. The SM-FeSV genome was rescued from the cells by superinfection with Rauscher leukemia virus (RLV), resulting in the release of SM-FeSV and RLV at a ratio of 1:1. This virus complex was used to infect NIH/3T3 cells at different times after infection. Hirt supernatants were prepared and viral DNA analyzed on analytical agarose gels followed by the Southern blotting technique and hybridization to viral cDNAs of mouse and feline origin. In parallel, DNA was extracted from clonal lines of both NRK and NIH/3T3 cells transformed by SM-FeSV. The DNA was subjected to restriction enzyme analysis, followed by Southern blotting and hybridization. These DNA preparations were also tested for their biological activity by transfection experiments using NIH/3T3 mouse fibroblasts as indicator cells.

Major Findings:

1. Unintegrated viral DNA extracted from NIH/3T3 cells infected with SM-FeSV (RLV) was analyzed on agarose gels followed by blotting and hybridization. Using MLV cDNA as a probe, we could detect a 9.5 kilobase pair (kbp) long linear form of RLV as well as two forms of supercoil molecules differing from each other in about 0.6 kbp. However, when we used FeLV cDNA as a probe, we could detect a major linear form of 6.5 kbp, most likely representing SM-FeSV proviral DNA. This DNA is now being isolated and subjected to restriction enzyme analysis.

2. High molecular weight DNAs extracted from SM-FeSV transformed NRK and NIH/3T3 cells were analyzed on agarose gels after digestion with various restriction enzymes. FeLV cDNA detected bands of 8.5 kbp and 8.0 kbp in Hind III digested SM-FeSV transformed rat and mouse DNAs, respectively. These DNAs are now being examined for their biological activity using transfection experiments on NIH/3T3 cells, prior to further purification of the fragments and subsequent cloning.

Significance to Biomedical Research and the Program of the Institute:

The cloning of SM-FeSV DNA will enable the isolation of the transforming gene of this virus and will provide an excellent tool for studies on the origin of feline sarcoma viruses and on the involvement of these viruses in tumor formation.

Proposed Course:

1. Cloning and restriction endonuclease mapping of the genome of SM-FeSV.

2. Mapping of the transforming region of the SM-FeSV genome by restriction endonuclease and transfection techniques.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05112-01-LCMB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Murine Leukemia Viruses: Mechanism of Leukemogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Storch	Research Associate	LCMB	NCI
OTHER:	S.A. Aaronson	Chief	LCMB	NCI
	M. Barbacid	Expert	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)

T.M. Chused	Senior Investigator	LMI	NIAID

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To understand how murine leukemia viruses cause lymphatic leukemia, recombinants have been isolated between a virus that causes B cell leukemia and viruses that cause T cell leukemia. Using fluorescent-labeled antibody against viral and lymphoid differentiation antigens and the fluorescence activated cell sorter, we will identify lymphoid subpopulations susceptible to infection and/or leukemogenesis by each parental or recombinant virus in order to map the region of viral genome responsible for transformation of specific lymphoid target cells.

PHS-6040
(Rev. 10-76)

Project Description

Objective:

To understand how murine leukemia viruses (MuLV) cause lymphatic leukemia, we will determine the region of the MuLV genome responsible for transformation of specific lymphoid cell targets.

Methods Employed and Course Taken:

We have used selective tissue culture conditions and temperature-sensitive MuLV mutants to isolate a series of recombinant viruses between a MuLV that causes a B cell leukemia and a virus that causes T cell leukemia. The origin of the viral sequences of each recombinant will be determined by immunotyping of its translational products and oligonucleotide fingerprinting of viral RNAs.

MuLV strains that cause T or B cell leukemias have been injected into inbred mouse strains. By fluorescence activated cell sorting techniques, we will determine those subpopulations of lymphoid cells that are susceptible to infection by each virus. The ability of the recombinants to cause leukemia of particular lymphocyte subpopulations will be correlated with mapping studies to determine the origin of their viral sequences.

Major Findings:

1. Potential recombinants between Kirsten-MuLV, which causes T cell leukemias, and Rauscher-MuLV, which causes B cell leukemias, have been isolated and cloned.

2. The initial target for infection by Moloney-MuLV and Rauscher-MuLV, appears to be undifferentiated lymphoid cells.

Significance to Biomedical Research and to the Program of the Institute:

Understanding of the mechanisms by which leukemia viruses transform specific lymphoid cell populations may provide important insights into alterations that lead to naturally occurring malignancies of these cell types.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05113-01-LCMB

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Characterization of the Immune Lymphocytes in Aging Normal and Tumored Animals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. Veronese	Visiting Fellow	LCMB NCI
Other:	G.J. Kelloff	Medical Researcher	LCMB NCI
	M. Kende	Expert	LOM NIDR
	R. Hill	Bio. Lab. Tech.	LCMB NCI

COOPERATING UNITS (if any)
J.R. Stephenson, LCMB NCI
I. Witz, Univ. of Tel Aviv
A. Nicolin, Univ. of Milan
M. Dinowitz, Micro. Assoc.

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Viral Immunology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has the long-range goal of prevention by immunological means of naturally occurring or chemically induced tumors in animal model systems that have viral, immunologic and epidemiologic similarities to human tumors. To this end, the project has defined the spontaneous tumor and endogenous type C virus expressions in colonies of untreated aging mice and has measured the host's natural and induced immune responses to same. Certain immunization regimens have been found by this project and other investigators to induce an immune response in the host that is effective in preventing experimentally-induced tumors and type C virus expressions. Therefore, the major emphasis of the project now is to specifically define the effector immune responses that occur and are responsible for the successful prevention of tumors, whether naturally occurring or induced. To achieve this, additional techniques specifically involving lymphocyte separation and characterization are being established. An equivalent emphasis is being placed on the nature and specificity of the target antigens that these immune responses are directed against, especially with regard to the gene products of the type C virus including the more recently defined transforming proteins.

Project Description

PROJECT A:

Objectives:

1. Examination of the cell surface reactivity of the 110,000-120,000 molecular weight polyprotein(s) of the feline sarcoma virus and Abelson leukemia virus in nonproducer transformed cells by antisera reactive with the nonstructural and structural components of these polyproteins.

2. Characterization of the host's cell-mediated and humoral immune responses to syngeneic nonproducer and producer transformed cells containing the 110,000-120,000 molecular weight polyproteins.

3. Immunoepidemiologic characterization of rats bearing tumors produced by various etiologic agents or occurring naturally to determine their immune reactivity with the transforming protein.

4. Production of hybridomas of the nonproducer rat cells to obtain high titered specific antibody to the transforming proteins. Such antibody to be used in diagnostic and therapeutic experimentation.

Methods Employed and Major Findings:

Syngeneic cell lines have been derived from embryonic cells of the inbred Fisher rat, thus providing a syngeneic system whereby the host's immune response could be examined against such cell lines productively and non-productively transformed by certain type C viruses. Cell lines under study include producer and nonproducer cells transformed by FeSV, Abelson LV, M-MSV. These various nonproducer clones differ in the types and levels of the gag structural proteins that are produced, but most lines under study, except certain controls, have the transforming proteins. These cell lines, as well as others carrying mutants of the type C viruses in mink cells, as well as rat cells, have been developed by Dr. John Stephenson. Experiments to date have shown that many of the weanling animals inoculated with non-producer transformed rat cells mount an immune response that results in regression of the tumor. Tumor dose kinetic studies are currently in progress. Initial studies reveal that a lymphocyte mediate cytotoxicity to syngeneic and allogeneic target cells containing the polyprotein is present in some of the inoculated animals. The time course of the CMI as it relates to tumor development and regression is under study. The humoral immune responses in these inoculated animals is under study. Further experimentation with antisera to the transforming protein is necessary to establish whether the protein is immunologically active at the cell surface in viable cells. The cell mediated and humoral immune responses of the rat challenged with syngeneic transformed cells will be examined against a variety of target cells in chromium release cytotoxicity assays. The specificity of the reactivities to the transforming protein in both the

Abelson and feline systems will be intensively studied as well as the host response to the type C virus structural proteins present in some of the inocula. Inocula will include producer as well as nonproducer transformed cells to determine which confers the best immune responses to the transforming protein as tested by both in vitro and in vivo assays. The lymphocyte and serum fractions responsible for the activity will be separated and characterized. Individual animals, regressors, and nonregressors will be compared in various immunologic assays for their activity to the transforming proteins. Hybridoma production will be attempted pending results of the planned studies.

PROJECT B:

Objectives:

1. Definition and characterization of immune suppressor cell activity in aging and tumored animals.

2. Lymphocyte characterization in the aging normal and tumored mouse based on receptors, surface markers and antibody dependence.

3. Definition of the target cell of the endogenous dual-tropic virus(es) from the aging BALB/c mouse.

Methods Employed and Major Findings:

Lymphoblast transformation assays have been adapted to measure suppressor cell activity present predominantly in the aging tumored mouse. Suppression of activity of specific populations of lymphocytes to specific viruses or viral antigens, including the transforming proteins, is under study. Lymphocyte separation techniques have been developed and are currently under study and in use. Successful separation of T, B and NK cells have been achieved and the immune function and reactivity of these subpopulations of lymphocytes to potential tumor antigens are under study. The target cells of the oncogenic dual-tropic viruses and the viruses' effect on the immune function is also under study in the BALB/c and C57/51 mouse.

PROJECTS A AND B:

Significance to Biomedical Research and the Progress of the Institute:

This effort has contributed significantly to an understanding of the endogenous type C virus relationship with its host and its genetic and epigenetic relationship to the host's naturally occurring tumors. It has aided the understanding of the host's immune response, natural and induced, to its spontaneous and induced tumors and to the viral gene products ubiquitously expressed in these tumors. Independent of etiologic relationships, these viral gene products appear to be acting as transplantation antigens in the tumors. The availability of transplantation antigens whose structural

specificities are defined have heretofore not generally been available which has slowed the progress of tumor transplantation research. With defined tumor transplantation antigens the effector immune responses in the host can now be systematically examined to determine their potential protective or enhancing effect on natural or induced tumors. A clear understanding of these immune mechanisms makes immunoprevention of spontaneously occurring and experimentally induced tumors a realistic goal to pursue.

Proposed Course:

Continuation of the above outlined studies.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05114-01-LCMB

PERIOD COVERED
October 1, 1979, to Setpember 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on Basic Mechanisms of Oncogenesis by Type C Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Blomberg	NIH Visiting Fellow	LCMB	NCI
Other:	J.R. Stephenson	Chief, Viral Genetics Sect.	LCMB	NCI
	R.P. Nalewaik	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

W.J.M. Van de Ven, FCRC	S. Oroszlan, FCRC
F.H. Reynolds, FCRC	G. Kelloff, LCMB NCI
R.V. Gilden, FCRC	A. Bernstein, Ont. Cancer Inst.

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION
Viral Genetics Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The involvement of humoral growth modulating systems acting at the cell surface in malignant transformation by sarcomagenic type C retroviruses was studied. It was found that transformation by Abelson leukemia virus abolished the binding of epidermal growth factor. A correlation between virus-associated protein kinase activity and loss of growth factor binding was also found.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To study the mechanism of transformation by type C viruses such as Abelson leukemia virus (AbLV) and feline sarcoma virus (FeSV), with special emphasis to their action at the cell surface.

Methods Employed:

Tissue culture, protein kinase labeling, immunoprecipitation, polyacrylamide gel electrophoresis, transfection, microinjection, DNA sequencing, restriction enzyme mapping.

Major Findings:

The role of humoral growth regulatory systems in transformation by sarcomagenic type C retroviruses was studied by investigating the number and affinity of EGF receptors on normal cells transformed by AbLV and FeSV, cells infected with td mutants of AbLV and revertant clones of AbLV transforming cells. It was found that morphological transformation always was combined with a loss of EGF binding sites. Additionally, a reciprocal relationship between protein kinase activity associated with the polyproteins, P120 and P115 of AbLV and FeSV, respectively, was present. This argues that a loss of EGF binding sites is directly or indirectly dependent on a functional transformation-related, virus-associated protein kinase. To further characterize this mechanism, we are currently studying the action of virus-associated protein kinases on the cell surface and their relation to the EGF receptor.

Significance to Biomedical Research and the Program of the Institute:

Uncovering the molecular mechanism of malignant transformation in cell culture will lead to a better understanding of carcinogenesis in humans and possibly give opportunities to intervene in the carcinogenetic process, or to selectively kill the cancer cells.

Proposed Course:

Transformation-related biochemical changes on the cell surface will be further studied.

Publications:

Blomberg, J., Reynolds, F.H. Jr., Van de Ven, W.J.M., and Stephenson, J.R.: Abelson murine leukemia virus transformation involves loss of epidermal growth factor binding sites. Nature, In Press.

SUMMARY REPORT

LABORATORY OF CHEMOPREVENTION

October 1, 1979 through September 30, 1980

Our laboratory has continued with its program of research, dealing with biological, biochemical, and pharmacological aspects of the ability of retinoids to control epithelial cell differentiation and to prevent the development of epithelial cancer. These studies have a two-fold purpose: from a basic scientific viewpoint, we wish to understand the mechanism of action of retinoids as completely as possible, while from a pragmatic, clinical viewpoint, we would like to see this field developed in support of the practical use of retinoids for prevention of cancer in men and women at high risk for development of malignancy. There is by now an extensive experimental literature that retinoids can be used effectively, both *in vitro* and *in vivo*, to suppress the phenotypic expression of malignancy caused by a variety of carcinogens, and further research in this area needs to be pursued.

Several different aspects of retinoid biology, biochemistry, and pharmacology have been studied in our laboratory. In the area of biology, we have studied the mechanism of the interaction between retinoids and a new growth factor (or factors) first isolated by Todaro and De Larco. This low molecular weight, highly stable polypeptide conveys properties of the transformed phenotype (growth in soft agar, release from density-dependent cell growth) on normal cells and its action can be blocked by very small doses of retinoids *in vitro*. In collaborative studies, with Drs. Todaro and De Larco, we are purifying the growth factor and examining the molecular and cellular basis whereby retinoids block its transforming action. Several epithelial tumors, as well as sarcomas, have been shown to be sources of the transforming factor. If a suitably pure protein can be isolated, we would like to determine its amino acid sequence, its physical properties, and the basis for the inhibitory effect that retinoids have on its mode of action.

In the area of biochemistry, we have carried out further studies of the metabolism of all-*trans*-retinoic acid and have further characterized several newly discovered metabolites. The metabolic interconversion of *trans*- and cis- metabolites has been studied in detail, to determine if isomerization to cis- forms is a required step before retinoids can exert their potent effects on epithelia. Synthetic retinoids, bearing substituent atoms on the 4-position of the ring, will be studied as a means to block degradative oxidative metabolism at this position, and thus to enhance or prolong the biological effectiveness of retinoids. Other biochemical studies will attempt to determine if an activated form of the retinoid molecule, beyond retinoic acid, can be isolated from any target tissue. At the present time, there is no compelling evidence that there is mandatory metabolic activation beyond the formation of retinoic acid.

In the area of pharmacology and toxicology, we have continued to investigate the role of the polar terminal group of the retinoid molecule in determining activity, metabolism, toxicity, and tissue distribution. Modification of this region of the molecule has generated a large number of analogs, some of which show markedly less toxic properties than all-trans-retinoic acid. The chemical basis for the lesser toxicity of the retinoids with modified polar terminal groups is not well understood. This problem has been investigated further, using the technique of fluorescence polarization to study the membrane-destabilizing effects of retinoids. It has been suggested that the toxic effects of retinoids may be mediated by their ability to increase the synthesis of prostaglandins, and interactions between retinoids and prostaglandins will also be investigated. Studies have also been conducted on the pharmacokinetic properties of retinoids with modified end groups, such as hydroxyphenylamide derivatives of retinoic acid and retinylidene-1,3-diketone derivatives. These are derivatives which have already been shown to be useful for suppression of the malignant phenotype, either in vitro or in vivo. These studies are defining the pattern of tissue distribution, metabolism, and excretion of these altered derivatives.

The problem of the isolation and characterization of transforming polypeptide growth factors (TGFs) is occupying an increasing amount of attention within our laboratory. Thus far, we have shown that TGFs can be isolated from a variety of epithelial and mesenchymal tumors of murine, chicken, and human origin, caused either by chemicals or viruses, or of spontaneous origin. All of these TGFs are acid-stable, low molecular weight materials, that will be the subject of future attempts at amino acid sequencing once they are purified to homogeneity. [New methods to achieve this desired purification have already been developed in our laboratory during the past year.] Based on the known properties of these TGFs, we will attempt to design new synthetic polypeptide inhibitors, which should represent a new class of chemopreventive agents.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO-1-CP-04494-04-LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Metabolism and Tissue Distribution Studies of Natural and Synthetic Retinoids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Anita B. Roberts Senior Staff Fellow LC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to increase our understanding of the metabolism of retinoids. Such information must be had before a rational approach can be taken to the design of new retinoids with greater activity in the reversal of preneoplasia in epithelial tissues. The investigation is focused on a study of the in vitro metabolism of retinoic acid and synthetic retinoids in subcellular systems of target tissues as compared to non-target tissues.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The purpose of this study is to investigate the metabolism of retinoids and from this to gain insight into the particular chemical modifications of synthetic retinoids which might enhance activity, and interfere with "deactivating" reactions which earmark a compound for elimination.

Methods Employed: An in vitro system for studying retinoid metabolism has been developed using a 10,000 x g supernatant preparation or microsomal fraction of hamster intestinal mucosa, liver, testis, and kidney. Ultracentrifugation is used to isolate subcellular fractions. Radioactive retinoids are used as substates.

The techniques employed for the detection of metabolites include freeze-drying, extraction, separation by high-pressure liquid chromatography (HPLC), and liquid scintillation counting.

Techniques for the study of the in vivo disposition of various retinoids involve an adaptation of a previously used extraction procedure to final analysis on HPLC.

Major Findings: It has been shown that the metabolism of retinoic acid (RA) in liver microsomes follows the scheme RA $\longrightarrow$ 4-hydroxy-RA $\longrightarrow$ 4-oxo-RA $\longrightarrow$ more polar metabolites. Attempts to demonstrate this reaction sequence in vivo have resulted in the identification of the 13-cis isomer of 4-oxo RA as an intermediate common to the metabolism of both 13-cis and all-trans-RA. These results show that isomerization of RA may be an important feature in its metabolism.

It can now be shown that in microsomes of the intestinal mucosa and testis, both presumably epithelial tissues of vitamin A, all-trans RA is metabolized to 13-cis-4-oxo RA, in agreement with the results obtained in vivo. In liver microsomes, on the other hand, all-trans-4-oxo RA is the principal product at RA concentrations greater than 10^{-8} M, but as the concentration of RA is lowered, increasing proportions of the 13-cis-4-oxo RA can be detected. These data suggest that metabolism of RA in the liver via the all-trans-isomeric route may comprise a detoxification mechanism for the rapid elimination of excesses of RA from the body, whereas target tissue metabolism via the 13-cis-isomeric route may represent a configurational activation, much as the isomerization of all-trans retinal to the 11-cis configuration is essential in the role of vitamin A in the visual process.

Significance to Biomedical Research and the Program of the Institute: A greater understanding of the metabolism of retinoids with the goal of identifying the reactive sites on the molecule, as well as defining the particular metabolic characteristics of the various target tissues, should greatly influence the design of new retinoids with better properties than the natural vitamin A compounds for the chemoprevention of carcinogenesis of epithelial tissues.

Proposed Course of Project: This project has been completed. Further work on retinoids will focus on their role in the chemoprevention of cancer, specifically on their interaction with polypeptide transforming growth factors.

Publications

Roberts, A.B., Nichols, M.D., Newton, D.L., and Sporn, M.B.: In vitro metabolism of retinoic acid in hamster intestine and liver. J. Biol. Chem., 254: 6296-6302, 1979.

Roberts, A.B., Frolik, C.A., Nichols, M.D., and Sporn, M.B.: Retinoid-dependent induction of the in vivo and in vitro metabolism of retinoic acid in tissues of the vitamin A-deficient hamster. J. Biol. Chem., 254: 6303-6309, 1979.

Frolik, C.A., Roberts, A.B., Tavela, T.E., Roller, P.R., Newton, D.L., and Sporn, M.B.: Isolation and identification of 4-hydroxy- and 4-keto-retinoic acid. In vitro metabolites of all-trans-retinoic acid in hamster trachea and liver. Biochemistry, 18: 2092-2097, 1979.

Roberts, A.B., and Frolik, C.A.: Recent advances in the in vivo and in vitro metabolism of retinoic acid. Fed. Proc., 38: 2524-2527, 1979.

Roberts, A.B., Lamb, L.C., and Sporn, M.B.: Metabolism of all-trans-retinoic acid in hamster liver microsomes: oxidation of 4-hydroxy- to 4-keto-retinoic acid. Arch. Biochem. Biophys. 199: 374-383, 1980.

Frolik, C.A., Roller, P.P., Roberts, A.B., and Sporn, M.B.: In vitro and in vivo metabolism of all-trans- and 13-cis-retinoic acid in hamsters. Identification of 13-cis-4-oxoretinoic acid. J. Biol. Chem. (in press.)

Roberts, A.B.:Microsomal oxidation of retinoic acid in hamster liver, intestine, and testis. Annals of the New York Acad. Sci. (in press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04549-08-LC

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Organ Culture Studies on Tracheobronchial Epithelium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael B. Sporn — Chief, Laboratory of Chemoprevention NCI

COOPERATING UNITS (if any)

Medicinal Chemistry Section; Laboratory of Chemistry, NIAMDD

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of vitamin A and its synthetic analogs (retinoids) to reverse a potentially premalignant lesion of tracheobronchial epithelium is being studied in organ culture. Keratinized, squamous metaplastic lesions are established in organ cultures of hamster tracheas. Addition of active retinoids causes reversal of these lesions and restoration of healthy mucociliary epithelium. Several new retinoids have been found to be extremely active and relatively free of toxicity, using this experimental system.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The principal objective is to study the direct effects of carcinogens and anti-carcinogenic agents (such as retinoids) on the cell population which is of immediate relevance for development of the most common form of human lung cancer, namely bronchogenic carcinoma. The principal experimental system that is being used measures the reversal of a potentially premalignant lesion, keratinized squamous metaplasia, by the addition of vitamin A and its synthetic analogs (retinoids).

Methods Employed: Classical methods of organ culture are being used. Tracheas are being obtained from either normal animals or from vitamin A-deficient animals. After lesions have become established in organ culture, the cultures are treated with different retinoids. The restoration of a normal ciliated and mucus-producing epithelium is then measured.

Major Findings: The optimal conditions for long-term organ culture of tracheobronchial epithelium of vitamin A-deficient hamsters have previously been defined, using medium CMRL-1066.

New synthetic retinoids, such as arotinoid analogs with two benzene rings in the molecule, tetrazole analogs of all-trans-retinamide, and tetrazole analogs of 13-cis-retinamide, have been found to be highly active compounds for reversal of keratinized squamous metaplasia.

Significance to Biomedical Research and the Program of the Institutes: The finding that new analogs of retinyl acetate, with modifications in the ring, the side chain and terminal portion of the molecule, are directly active on the tracheal epithelium is particularly important. This finding opens the way for synthesis of new forms of vitamin A that may have greater pharmacological usefulness in man for prevention of cancer. Studies are currently in progress in the Laboratory of Chemoprevention to evaluate the direct effects of synthetic retinoids on preneoplastic mammary epithelium of rats.

Proposed Course of Project: To continue as outlined above.

Publications

Moon, R.C., Thompson, H.J., Becci, P.J., Grubbs, C.J., Gander, R.J., Newton, D.L., Smith, J.M., Phillips, S.L., Henderson, W.R., Mullen, L.T., Brown, C.C., Sporn, M.B.: 4-Hydroxyphenyl retinamide, a new retinoid for prevention of breast cancer in the experimental animal. Cancer Res. 39: 1339-1346, 1979.

Frolik, C.A., Roberts, A.B., Tavela, T.E., Roller, P.P., Newton, D.L., and Sporn, M.B.: Isolation and identification of 4-hydroxy- and 4-oxoretinoic acid. In vitro metabolites of all-trans-retinoic acid in hamster trachea and liver. Biochemistry 18: 2092-2097, 1979.

Sporn, M.B.: Retinoids and cancer prevention. In Slaga, T.J. (Ed.): Modifiers of Chemical Carcinogenesis. New York, Raven Press, 1979, pp. 99-109.

Roberts, A.B., Frolik, C.A., Newton, D.L., Smith, J.M., and Sporn, M.B.: Role of retinoids in prevention of carcinogenesis. Adv. Pharmacol. Therapeut. 8: 5-14, 1979.

Roberts, A.B., Nichols, M.D., Newton, D.L., and Sporn, M.B.: In vitro metabolism of retinoic acid in hamster intestine and liver. J. Biol. Chem. 254: 6296-6302, 1979.

Roberts, A.B., Frolik, C.A., Nichols, M.D., Sporn, M.B.: Retinoid-dependent induction of the in vivo and in vitro metabolism of retinoic acid in tissues of the vitamin A-deficient hamster. J. Biol. Chem. 254: 6303-309, 1979.

Sporn, M.B., and Newton, D.L.: Chemoprevention of cancer with retinoids. Fed. Proc. 38: 2528-2534, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO-1-CP-05051-02-LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Isolation of Polypeptide Transforming Factors from Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Anita B. Roberts Senior Staff Fellow: LC NCI

COOPERATING UNITS (if any)
Laboratory of Viral Carcinogenesis, Drs. George J. Todaro and Joseph E. De Larco

LAB/BRANCH
Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop methods for the extraction, isolation, and characterization of polypeptides with the property of conferring the transformed phenotype on normal indicator cells. These purified transforming growth factors (TGFs) will then form the basis for investigation of the mechanism of transformation and more specifically for the development of protein antagonists to these TGFs. Central to the investigation is the purification and characterization of the sarcoma growth factors (SGFs) found upon direct extraction of murine-sarcoma virus transformed cells grown in culture. Once isolated, the generality of this SGF will then be investigated by comparing its properties to those of TGFs isolated from a variety of tumor cells of non-viral origin, as well as to those of polypeptides with similar characteristics isolated from normal mouse tissues.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The ultimate goal of this research is to determine the complete amino acid sequence of a transforming growth factor (TGF) and then to use this as a basis for the design of antagonists to these polypeptides. Once the protein is purified, it is our intention to investigate the mechanism of action of these TGF's in the carcinogenic transformation of cells as well as the mechanism of their inhibition by potential synthetic polypeptide antagnoists or natural antagonists such as the retinoids, which are known to have anti-promoter activity.

Methods Employed: Classical methods of protein purification and new methods based on reverse-phase-high-pressure liquid chromatography will be employed. These will include dialysis, solvent precipitation, the use of sizing gels and ion-exchange columns, as well as methods based on the electrophoretic properties of these polypeptides.

With the help of Dr. Joseph E. De Larco (Meloy Laboratories), an assay for these TGF's based on their ability to cause a normal anchorage-dependent indicator cell to grow in an anchorage-independent manner (as assessed by growth in semi-solid agar medium) has been set up in our laboratory and is routinely being used to monitor the purification of these TGF's.

Major Findings: Polypeptides characterized by their ability to confer a transformed phenotype on an untransformed indicator cell have been isolated directly from tumor cells growing both in culture and in the animal, using an acid-ethanol extraction procedure. Assay of these polypeptides is based on their ability to induce normal rat kidney fibroblasts to form colonies in soft agar. Peptides from murine sarcoma virus-transformed mouse 3T3 cells grown in culture had the highest specific activity in this assay; peptides from sarcomas produced from these cells or from chemically-induced transplantable bladder carcinomas of mice were one-third as active, and peptides from a chemically-induced rat tracheal carcinoma had only one-tenth the activity. Treatment with either trypsin or dithiothreitol destroyed the activity of all of these materials. Thus these intracellular polypeptides from both virally and chemically-transformed cells have properties similar to those described for the sarcoma growth factors (SGFs) previously isolated by De Larco and Todaro from the conditioned medium of sarcoma virus-transformed mouse 3T3 cells, suggesting the definition of a new class of transforming growth factors (TGFs) common to tumor cells of different origin. The transforming peptides from the cultured sarcoma virus-infected cells were separated by gel filtration into two fractions of apparent molecular weight 7000 and 10000. The major fraction at 7000 M.W. represented approximately 0.1 percent of the original cell protein and had a specific activity 50-fold that of the original acid-ethanol extract.

More recently it has been found that acid-ethanol extracts of normal mouse tissues also contain TGF-like activity. The greatest tissue concentration of these factors is found in the submaxillary glands of either male or female mice. Upon gel filtraction in 1 M acetic acid, these polypeptides have an apparent molecular weight of 4000 - 10,000 and can be shown to be distinct from epidermal growth factor which is also efficiently extracted by this procedure. Their relationships to the TGF's of tumor cells is being explored.

Significance to Biomedical Research and the Program of the Institute: The discovery of TGFs which act as direct effectors of phenotypic transformation, together with the discovery that retinoids can block the effect of SGF on cells, offers a unique opportunity to explore the mechanism of control of the carcinogenic process. Eventually, it is hoped that a better approach to the chemoprevention of carcinogenesis will develop from the results of these investigations.

Proposed Course of the Project: Future work will center around the purification of the intracellular SGF from murine-sarcoma virus-infected mouse 3T3 cells. Once purified, it is hoped that similar methods can be used to purify other TGFs and that their familial relationships can then be explored. In addition, we hope to explore the cellular controls on the synthesis and excretion of these TGFs, the nature of the membrane receptors for these TGFs, as well as the possibility of blocking the expression of the biological activity of these TGFs by the use of synthetic or natural antagonists.

Publications

Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., De Larco, J.E., and Todaro, G.J.:Transforming growth factors; Isolation of polypeptides from virally and chemically transformed cells by acid-ethanol extraction. Proc. Nat. Acad Sci., 1980 (in press.)

Sporn, M.B., Newton, D.L., Roberts, A.B., De Larco, J.E., and Todaro, G.J.: Retinoids and the supression of the effects of polypeptide transforming factors - a new molecular approach to the chemoprevention of cancer. In Sartorelli, A.C., Bertino, J.R., and Lazo, J.S. (Eds.): Molecular Actions and Targets for Cancer Chemotherapeutic Agents. Academic Press, 1980 (in press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05052-02-LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Control of Epithelial Differentiation and Carcinogenesis by vitamin A

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C.A. Frolik Chemist: LC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.7	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to find an agent that would cause a reversal of preneoplasia in epithelial tissue. Retinoids (vitamin A and its analogs) have been the compounds initially being investigated. Emphasis has been placed on determining the metabolic pathways of all-trans-retinyl acetate and all-trans- and 13-cis-retinoic acid. Retinoic acid has been shown to be metabolized to several more polar metabolites both in vitro in a hamster tracheal organ culture system and in vivo in the normal hamster and rat. Several of these metabolites have been identified as the all-trans- and 13-cis- isomers of 4-hydroxy- and 4-oxoretinoic acid. A comparison of the metabolism of all-trans- and 13-cis-retinoic acid demonstrates that these two isomers may to some extent be sharing a common metabolic pathway that includes 13-cis-4-oxoretinoic acid. Although a knowledge of the metabolic pathway of retinoic acid would help to decipher the biochemical mechanism of action of vitamin A in controlling epithelial cell differentiation and would aid in designing analogs of vitamin A that would display biological activity in the reversal of preneoplasia, no metabolites have yet been found that show a biological activity greater than the parent retinoic acid.

Project Description

Objectives: It is the purpose of this project to determine the role of vitamin A in the control of both normal and carcinogenic epithelial cell differentiation. Special emphasis has been placed on studying the metabolic pathway of both natural and synthetic retinoids in an effort to determine possible sites of activation or deactivation on the retinoid molecule. This information can then be used in the design of compounds that will display high biological activity in the reversal of preneoplasia while also showing low levels of toxicity.

Methods Employed: The in vitro metabolism of several retinoids has been investigated in hamster tracheal organ cultures and in hamster liver 10,000 x g supernatant preparations. In addition, the biliary excretion of all-trans- and 13-cis-retinoic acid and their metabolites has been examined in vitamin A normal rats (in joint effort with Dr. Brian Swanson of the Laboratory of Chemoprevention). The in vitro or in vivo metabolites are extracted into an organic phase utilizing a lyophilization procedure and are further separated by reverse-phase high-pressure liquid chromatography. For identification purposes the metabolites are methylated and rechromatographed on normal-phase high-pressure liquid chromatographic columns prior to comparison with standard compounds based on ultraviolet, mass spectral, nuclear magnetic resonance and chromatographic characteristics.

Major Findings: Previous investigations in this laboratory have shown that all-trans-retinoic acid is metabolized in vitro to several more polar compounds including all-trans-4-hydroxy- and 4-oxoretinoic acid. Recent findings,however, now suggest that at physiological levels both all-trans- and 13-cis-retinoic acid are metabolized in vivo to 13-cis-4-hydroxy- and 4-oxoretinoic acid with the all-trans- isomers being important only at pharmacological dose levels. Attack at the four position of the retinoid molecule is currently believed to be the first step in elimination of retinoic acid from the body. Other elimination pathways have been shown to involve glucuronide formation. The presence of all-trans-retinoyl- -glucuronide has been confirmed in the bile of rats given all-trans-retinoic acid while 13-cis-retinoyl- -glucuronide as well as 13-cis-4-oxoretinoyl- - glucuronide have been identified in the bile of rats administered 13-cis-retinoic acid. Finally, all-trans-retinyl acetate has been shown to be metabolized to all-trans-retinoic acid in a hamster tracheal organ culture system supporting the idea that retinol may have to be converted to retinoic acid in order to function in the control of epithelial differentiation.

Significance to Biomedical Research and the Program of the Institute: A knowledge of the activation and/or inactivation sites on the retinoid molecule would provide useful information that would aid in the development of an analog of vitamin A that would display biological activity in the reversal of preneoplasia in epithelial tissues and yet show little or no toxicity in the whole animal.

Proposed Course of the Project: Because no active metabolites of retinoic acid have yet been found and since the initial steps in the elimination pathway of retinoic acid from the body have been elucidated, further work in our laboratory will concentrate on other methods of chemoprevention of cancer. This project will therefore be terminated by the end of the current report period.

Publications

Frolik, C.A., Roberts, A.B., Tavela, T.E., Roller, P.P., Newton, D.L., and Sporn, M.B.: Isolation and identification of 4-hydroxy- and 4-oxo-retinoic acid. In vitro metabolites of all-trans-retinoic acid in hamster trachea and liver. Biochemistry. 18: 2092-2097, 1979.

Roberts, A.B., Frolik, C.A., Nichols, M.D., and Sporn, M.B.: Retinoid-dependent induction of the in vivo and in vitro metabolism of retinoic acid in tissues of the vitamin A-deficient hamster. J. Biol. Chem. 254: 6303-6309, 1979.

Roberts, A.B. and Frolik, C.A.: Recent advances in the in vivo and in vitro metabolism of retinoic acid. Federation Proc. 38: 2524-2527, 1979.

Swanson, B.N., Frolik, C.A., Zaharevitz, D.W., Roller, P.P., and Sporn, M.B.: Dose-dependent kinetics of all-trans-retinoic acid in rats: Plasma levels and excretion into bile, urine and feces. Biochem. Pharmacol. (in press.)

Frolik, C.A., Roller, P.P., Roberts, A.B., and Sporn, M.B.: In vitro and in vivo metabolism of all-trans- and 13-cis-retinoic acid in hamsters. Identification of 13-cis-4-oxoretinoic acid. J. Biol. Chem. (in press.)

Frolik, C.A.: In vitro and in vivo metabolism of all-trans- and 13-cis-retinoic acid in the hamster. N.Y. Acad. of Sci. (in press.)

Frolik, C.A. and Roller, P.P.: The role of retinoids (vitamin A and its analogs) in the prevention of epithelial cancer. In Aszalos, A. (Ed.): Antitumor Compounds of Natural Origin. Boca Raton, FL, CRC Press (in press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z0-1-CP-05053-02-LC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on the Mechanism of Toxicity of Natural and Synthetic Retinoids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert Meeks Staff Fellow LC NCI

COOPERATING UNITS (if any)

none

LAB/BRANCH
Laboratory of Chemoprevention

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) It has been reported that (1) retinoids can destabilize membranes, especially lysosomal membranes with the possible subsequent release of hydrolytic enzymes (2) retinoids can increase the fluidity of membranes in a dose-related manner (3) retinoids can possibly form free radicals by the nature of their structure, and (4) retinoic acid can be oxidatively decarboxylated in vitro using incubation conditions similar to those used for the NADPH stimulated peroxidation of microsomal membranes.

Based upon this observation studies were undertaken to examine electron donating and electron accepting properties of selected retinoids and to try to correlate these properties to the ability of retinoids to affect membrane peroxidation which, in turn, could be correlated to the ability of retinoids to destabilize membranes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The object of this study was to examine the electron-donating and -accepting properties of various retinoids and to then examine the ability of these selected retinoids to stimulate or inhibit the peroxidation of membrane lipids. The object was to then demonstrate that the peroxidation of membrane lipids could account for the changes in microviscosity observed when membranes are treated with retinoids. This would then give some insight as to the mechanism of toxicity of retinoids and would then allow for a rational approach to the development of synthetic synthetic retinoids which have decreased toxicity.

Methods Employed: Electron-donating studies were conducted using 7,7,7,8-tetracyanoquinodimethan (TCNQ in either acetonitrile or dimethylformamide (DMF). Under appropriate conditions TCNQ will accept an electron to form the TCNQ-radical which has a characteristic absorption band at 842 nm. Increasing concentrations of retinoids were placed in solvent containing TCNQ, and the rate of formation of TCNQ was measured spectrophotometrically and the rate of disappearance of retinoid was measured using HPLC.

The effect of retinoids on the peroxidation of membrane lipids was studied using a system similar to the one used for the NADPH dependent peroxidation of microsomal membranes which is also similar to the same system used for the oxidative decarboxylation of retinoid acid. The extent of membrane peroxidation was determined by using the 2-thiobarbituric acid method which measures malonyldialdehyde, a product of peroxidation.

Major Findings: When retinol was added to the TCNQ in DMF, an increase in TCNQ was observed and appeared to follow the rate law

$$\frac{d\ [TCNQ^-]}{dt} = k_i[R.]_o^{1/2}([TCNQ]_o-[TCNQ^-])^{1/2} - R_2[R]^{1/2}[TCNQ^-]^2$$

where k = the rate constants R = retinol

The disappearance of retinol as followed by HPLC was strictly first order and followed the rate law

$$\frac{d[R]}{dt} = -k[R]$$

The concentration of retinol was not in any way related to the concentration of TCNQ since the retinol was gone long before the TCNQ-approached its final value indicating the possibility of the formation of a charge transfer complex in the reaction mechanism.

When the retinoic acid was placed in this sytem, there seemed to be a linear dependence of the rate of formation on $TCNQ^-$ with the concentraction of retinoic acid. In addition, the concentraction of retinoic acid necessary to stimulate the formation of $TCNQ^-$ was 10-12 times the concentrction of retinol necessary.

The effect of retinoids on membrane peroxidation was the opposite of the effects expected. It was postulated that low doses of retinoids would act as anti-oxidants and that high doses would act as pro-oxidants and would stimulate peroxidation. This was not the case, as the concentration of retinol or retinoic acid was increased, the peroxidation of membrane lipids was inhibited 70%. However, if the cofactors (containing NADPH, mg^{2+}, Fe^{2+}, and were incubated with retinoid alone, peroxidation was enhanced by 50%. The presence of DPPD blocked the peroxidation of membrane lipids in both systems.

Significance to Biomedical Research and the Program of the Institute: By comparing and contrasting the structures of the various retinoids for the ability to act as pro- and anti-oxidants we are beginning to gain insight as to the possible mechanism of toxicity of retinoids. Armed with this knowledge, we can greatly influence the design of new retinoids with decreased toxicological activity.

Proposed Course of Project: This project has been terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z0-1-CP-05054-02-LC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pharmacokinetics and Metabolism of Retinoids in Rodents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Brian Swanson Staff Fellow LC NCI

COOPERATING UNITS (if any)

Carcinogen Metabolism and Toxicology, Dr. Peter P. Roller

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/3	1/3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on the pharmacokinetics and metabolism of retinoids were continued. Unusual, dose-dependent plasma clearance and metabolism of all-trans-retinoic acid were extensively investigated. The possibility of enzymatic hydrolysis of amides of retinoic acid was also examined.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: All-trans-retinoic acid is a biologically active metabolite of retinol (vitamin A_1). Previous laboratory studies on this metabolite have utilized a wide range of doses both in vivo and in vitro. The possibility of dose-dependent changes in the rate of clearance and in the metabolic fate of all-trans-retinoic acid has not been investigated. It has been the objective of our laboratory to examine this possibility in rats using the latest analytical techniques.

Numerous amide derivatives of all-trans-retinoic acid have been synthesized recently. The biological activity of these amides may be due to metabolism of the amides to active metabolites, such as all-trans-retinoic acid. One of our objectives has been to determine the extent of enzymatic hydrolysis of amides of retinoic acid.

Methods Employed: All-trans-retinoic acid and its amides were infused intravenously into rats. Bile and blood samples were collected using intraluminal catheters. Animals were maintained in metabolism cages so that urine and feces could be obtained as well. Radioisotopes of the retinoids were used frequently in order to facilitate isolation and identification of metabolites and to increase assay sensitivity for individual retinoids. High-pressure liquid chromatography (HPLC) was used to separate retinoids and their metabolites from each other and from substances in biological fluids. HPLC was also used to purify metabolites prior to ultraviolet and mass spectral analysis. Liquid scintillation spectrophotometry was used for measuring radioactivity in feces, urine, bile and HPLC eluate fractions. Enzymatic and chemical hydrolysis of glucuronide metabolites was accomplished with β glucuronidase and sodium hydroxide, respectively. Diazomethane was used for methylating retinoid metabolites.

Major Findings: Plasma concentration-time curves for all-trans-retinoic acid.

The dose-dependent kinetics of this retinoid in plasma was not the result of enterohepatic recirculation of the the intact compound, since retinoid acid levels in plasma are not lower in rats with biliary fistulas given comparable doses. Hydrolysis of the amide bond in N-(hydroxyphenyl)-all-trans-retinamide was found to be a minor or nonexistent metabolic reaction in vivo.

Significance to Biomedical Research and the Program of the Institute: Our studies have accurately described the kinetics of all-trans-retinoic acid in rat plasma after a wide range of intravenous doses. This information can be used for explaining the relative biological efficacy of certain dosage schedules of all-trans-retinoic acid that have been used in the past and for assisting in the construction of future dosage regimens. For the first time, saturation kinetics have been established for a retinoid in vivo. The possibility of non-first order kinetics for clinically-used retinoids should probably be investigated in the future in man.

Publications

Swanson, B.N., Zaharevitz, D.W., Sporn, M.B.: Kinetics of N-(4-hydroxyphenyl)-all-trans-retinamide in rats. Drug Metab. and Disp., 1980 (in press.)

Swanson, B.N., Frolik, C.A., Zaharevitz, D.W., Roller, P.P., and Sporn, M.B.: Dose-dependent kinetics of all-trans-retinoic-acid rats: plasma levels and excretion into bile, urine, and feces Biochem. Pharmacol. (in press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05116-01-CP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Characterization of a Polypeptide Transforming Factor from Human Tumor Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles A. Frolik Chemist: LC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Acid stable polypeptides have been isolated from tumor cells grown both in culture and in the animal. (Roberts et. al. Proc. Natl. Acad. Sci, in press) These proteins are able to produce a reversible morphological transformation of normal rat kidney fibroblast cells that allows them to grow in a soft agar medium where normal cell growth is prevented. It is the prupose of this project to ascertain whether such transforming growth factors are present in human tumor cells and the role these factors may play in the process of carcinogenesis. Initial investigation will concentrate on determination of the amount of such transforming growth factors in several different human cell lines and their properties with a special emphasis on purification and physical characterization. Once these proteins have been characterized, analogs will be synthesized and tested for their ability to inhibit the transforming activity of the natural peptide and therefore, possibly, inhibit the carcinogenic process. Finally, the mechanism whereby these factors interact with the cell to cause the phenotypic cell transformation will be investigated in the hope of offering a greater understanding of carcinogenesis itself.

Project Description

Objectives: This is a new project. The purpose of this study is to explore new methods that may be useful in the chemoprevention of cancer. Special emphasis will initially be placed on the isolation and characterization of acid stable polypeptide transforming growth factors (TGFs) found in various human tumor cells. Once characterized, TGF analogs will be synthesized and tested for their ability to block the action of TGF in causing phenotypic cell transformation.

Methods Employed: TGF is extracted from various samples using an acid-ethanol procedure recently developed by Dr. Anita Roberts in our laboratory and based on the insulin isolation procedure of Davoren. In addition, an extraction procedure based on the binding of peptides to octadecasilyl-silica is being investigated. Once extracted the peptides will be further purified by a combination of the classical methods including use of sizing gels, ion exchange columns and polyacrylamide gel electrophoresis with the more recently developed method of high-pressure liquid chromatography of proteins. Transforming activity is being determined by a soft agar growth assay.

Major Findings: Various human tumor cell lines have been investigated for the presence of transforming activity. To date, such activity has been observed in all strains examined. The highest activity is found in the intestinal tumor line, HeLa, and in a fibrosarcoma and a rhabdomyosarcoma line. In addition, TGF has been detected in two breast cell lines, 2 leukemia lines, and in a metastatic melanoma cell line.

Significance to Biomedical Research and the Program of the Institute: Through a knowledge of the physical structure of TGF and its mode of action in causing phenotypic transformation, it is hoped that a sound approach to the chemoprevention of carcinogenesis will be able to be developed.

Proposed Course of the Project: In the future, work will initially center around the purification and characterization of the transforming factors and could possibly include, as well, initial investigatons into the mode of action of TGF in causing a reversible morphological transformation of normal cells.

SUMMARY REPORT

LABORATORY OF EXPERIMENTAL PATHOLOGY

October 1, 1979 through September 30, 1980

The Laboratory of Experimental Pathology "plans, develops and implements a research program on the experimental pathology of carcinogenesis, involving: (1) development, characterization and evaluation of experimental models of human cancer using in vivo and in vitro carcinogenesis methods; (2) experimental study of the pathogenesis of cancer in human tissues; (3) morphological and biochemical studies on chemical carcinogenesis and its inhibition in selected animal, tissue and cellular models; (4) research on the pathogenesis of cancer related to perinatal exposures; and (5) studies of biochemical mechanisms involved in the expression of the transformed state".

The Laboratory consists of the Office of the Chief and four Sections: "Differentiation Control", "Human Tissue Studies", "In Vitro Pathogenesis" and "Perinatal Carcinogenesis." Investigators in different sections collaborate in their studies on mechanisms and models of carcinogenesis.

The main focus of investigations in the Laboratory is on the study of the pathogenesis of chemically induced neoplastic disease using morphological and biochemical approaches at all levels of biological organization, including human tissues, animal models, organ and cell cultures as well as molecular interactions.

Particular emphasis is given to studies of chemical carcinogenesis in lining epithelia which are the tissues of origin of most human cancers.

There is a fundamental need to relate the process of carcinogenesis to the specific characteristics of the tissues from which the induced tumors originate. Experimental chemical carcinogenesis is the result of a chemical-biological interaction resulting in pathologic responses typical of the different tissues and cells of origin. Human cancer is characterized by a similarly wide variety of pathologic response patterns.

Numerous animal models for major forms of human cancer, especially those of epithelial origin, were developed in recent years. Work from this Laboratory contributed to the establishment and investigation of biological models for cancers of the epidermis, respiratory tract, digestive tract, pancreas, liver, kidney, mammary gland, endocrine system, central and peripheral nervous system, and others. These models became the tools for studying the pathogenesis of major types of cancer. Such studies have rapidly advanced by combining both morphological and biochemical methods, focused on the target tissues, and by correlating studies at the organ and tissue levels with those at the cellular and molecular levels.

Most of the current knowledge of carcinogenesis comes from studies in animals, and a great need exists to correlate animal with human pathology. This is a crucial problem in the interpretation of research findings in terms of human cancer pathogenesis and also in the assessment of carcinogenic risks for humans on the basis of experimental studies.

A broad range of experimental pathology methods are being used for the study of chemical carcinogenesis, focussing on the sequence of events leading from the activation of carcinogens and their interaction with cellular macromolecules to the induction of a transformed state and to its expression in cells, tissues and the whole organism. Areas of special emphasis that unify different experimental approaches in the Laboratory include: (a) the study of carcinogenesis in epithelial tissues; (b) the correlation of different levels of biological organization and of different species including humans; (c) the study of mechanisms that modify the carcinogenic response (inhibition, promotion, synergism); and (d) the identification of markers of the interaction of carcinogens with target cells. This comprehensive approach to selected problems of cancer pathogenesis is designed to provide knowledge directly relevant to the understanding of the causation of human cancers and their prevention.

A summary of the current activities and accomplishments of the Laboratory is given in the following reports.

OFFICE OF THE CHIEF - In addition to overall coordination of the Laboratory, the following activities were contributed by the research staff of this Office.

Criteria for the identification and evaluation of carcinogenic agents and for risk assessment were analyzed and defined; documentation was assembled and reviewed on carcinogenic effects of environmental chemicals and potential occupational carcinogens; specific fields of environmental carcinogenesis research were reviewed, including occupational carcinogens, pesticides and metals. In collaboration with the Interagency Regulatory Liaison Group further review was conducted of the criteria, accepted in 1979 by the relevant federal agencies, for the identification of potential carcinogens and the estimation of their risks. In collaboration with the U. S. Regulatory Council, a report was prepared outlining a national policy on the regulation of carcinogens; this report, approved by all relevant federal agencies was published in October 1979.

In collaboration with the Occupational Safety and Health Administration, extensive review and advice were contributed in the preparation of the OSHA policy on the regulation of potential occupational carcinogens, issued in January 1980.

Other collaboration was given to the Environmental Protection Agency (EPA), National Institute on Occupational Safety and Health (NIOSH), Consumer Product Safety Commission (CPSC) and other federal agencies.

Extensive bibliographic documentation was assembled on dose-response relationships and on synergistic effects in carcinogenesis studies.

Laboratory studies were continued on dose-response relationships of carcinogens in mutagenesis and cell transformation assays and on the effects of concurrent exposure to multiple carcinogens. Using the Ames Salmonella assay, with or without metabolic activation, several combinations of carcinogens were studied in a large range of dose levels; studies using low levels of exposure showed clear examples of additive, synergistic and inhibitory interactions in mutagenesis: polycyclic aromatic hydrocarbons were mutually inhibitory, aromatic amines were mostly additive with examples of inhibitory and synergistic combinations, while a group of structrually different carcinogens was found to act synergistically.

A mouse fibroblast cell line, Balb/c 3T3 clone 31, was set up and used for neoplastic transformation assays in studies on dose-response and combined effects of carcinogens parallel to those for mutagenesis.

DIFFERENTIATION CONTROL SECTION: "(1) Studies the biochemical mode of action of vitamin A and its derivatives, the retinoids, in controlling and modulating the expression of the normal and the neoplastically-transformed state; (2) conducts investigations of the metabolism of retinol and retinoic acid in normal and transformed cells; (3) studies cell-surface glycoproteins involved in cell adhesion and control of growth."

Effects on adhesion and EGF-receptor sites: Recent studies have demonstrated that retinoids with vitamin A activity in other systems are also active in increasing the adhesive properties of a variety of fibroblastic murine cell lines, including Balb/c 3T3, 3T6 and 3T12 cells. In this past year, retinoids have also been shown to increase specifically the number of available receptor sites for epidermal growth factors (EGF) in all these cell lines, from 6 to 10 fold above control levels. Such an increase occurs within hours after administration of the active retinoid at concentration of 10^{-8}M or higher. The phenomenon is specific, inasmuch as receptor sites for insulin are not affected under the same treatment schedule. A small increase of 20 to 40% above control was observed for concanavalin A receptor sites.

The enhancement of the number of EGF receptor sites was common to a variety of cell lines. The few which did not respond were those with the highest number of EGF-receptors. The kinetics of the response paralleled those of the increase in adhesion and in sulphated glycosaminoglycans.

Cell-surface glycoprotein changes: Inasmuch as these responses to active retinoids all appear to implicate changes at the level of the cell surface, research was conducted to characterize retinoid-induced alterations in glycosylated cell surface components of retinoic acid-treated and control 3T12 fibroblasts. The use of highly specific exo- and endoglycosidases aided in the definition of a specific effect of retinoids on glycoproteins of the cell surface; the synthesis of these glycoproteins was increased preferentially compared to whole cell glycoproteins, after treatment with RA. Whole cellular glycopeptides labeled with [2-^{3}H]mannose were shown to be prevalently of the "polymannose type", i.e. the type containing 6 to 7 mannosyl residues attached to Di-N-acetylchitobiose-asparagine, whereas the cell surface glycopeptides labeled with fucose as well as mannose were shown to be almost exclusively of the "complex type", i.e. the type containing N-acetylneuraminic acid, galactose, glucosamine, mannose, and fucose linked to Di-N-acetylchitobiose-asparagine.

Development of new high pressure liquid chromatography (HPLC) systems for the analysis of retinoids, including retinylphosphate and mannosyl retinylphosphate. A new reverse phase ion pair HPLC procedure for the separation of retinoids was developed. This procedure allows the separation of derivatives of vitamin A in one chromatographic step and in order of decreasing polarity.

Metabolic studies on 13-cis and all-trans retinoic acid in normal Sprague-Dawley rats has demonstrated an interconversion of these two products. Only trace amounts of these compounds were detected after 3 hours from

the injection of the labeled precursor. Most of the label cochromatographed in the area of highly polar derivatives, at least in liver, testis, and intestinal tissue.

New developments in studies on the biosynthesis of mannosylretinylphosphate. Mannosylretinylphosphate (MRP) is a biosynthetic product of a variety of tissues in vivo and of their microsomal membranes in vitro. In addition to this mannolipid, 3T12 cells were also found to synthesize a similar compound containing a metabolite of retinoic acid and termed mannosylretinoid phosphate or metabolite I.

Thus, three mannolipids have been isolated, MRP, metabolite I and dolichylmannosylphosphate (DMP). The specific function of these compounds in glycoprotein synthesis remains to be clarified. Precursor-product studies in cell-free systems are unlikely to yield useful information because the disrupted membranes catalyze the synthesis of $Man^{\alpha 1,2}Man$, $Man^{\alpha 1,3}Man$ and $Man^{\alpha 1,6}Man$ from both precursors, MRP and DMP, indiscriminately. The lack of substrate specificity of the mannosyl transferases, therefore, rendered this approach of limited usefulness. A breakthrough of considerable interest resulted from the observation that a whole membrane fraction from the lymphoma cell line Thy-1^+ does not synthesize MRP from exogenous retinylphosphate, whereas, it is capable of synthesizing DMP, and a mannolipid of the same chromatographic characteristics as metabolite I of retinoic acid.

Moreover, a mutant of this lymphoma cell line, the Thy E^- was isolated by Throwbridge and Hyman and shown not to be defective in the synthesis of DMP. In addition, we found that the mutant (as the wildtype), is also defective in the synthesis of MRP. The only mannolipid made by the whole membrane fraction from Thy E appears to have the same chromatographic characteristics as metabolite I. Thus, in conclusion, these systems of lymphoma cell lines represent the only ones known to lack either the GDP-mannose-retinylphosphate mannosyltransferase (Enzyme I) in the wildtype, or both the GDP-mannosedolichylphosphate mannosyltransferase (Enzyme II) as well as Enzyme I in the mutant. This development should permit the characterization of the precise step in mannosylation reactions in which MRP, DMP, and possibly metabolite I are involved.

HUMAN TISSUE STUDIES SECTION - "(1) Conducts carcinogenesis studies on the pathogenesis of human cancer, especially carcinomas, using tissues from human target organs and from experimental animals, by organ culture and related methods; (2) conducts cellular and molecular investigations concerning both the function and differentiation on human epithelial cells and the interactions between carcinogens and these epithelial cells; (3) conducts experimental and clinical investigations to investigate the mechanism(s) of variation in carcinogenic susceptibility among people; and (4) investigates the problem of extrapolation of carcinogenesis data among species."

Model systems for human carcinogenesis: Carcinogenesis researchers have striven for decades to interpret data obtained from animal studies in terms of their applicability to humans. The carcinogenic hazard of environmental chemicals has been determined primarily from studies with experimental animals and from retrospective epidemiological investigations. An experimental approach to the direct study of carcinogenesis in human tissues and cells has been recently developed to assess the: a) mechanisms of carcinogenesis in human cells; b) variation of carcinogenic susceptibility among individuals; and c) validity of the extrapolation of carcinogenesis data from experimental animals to the human situation. Studies of carcinogenesis can now be conducted directly in human target tissues. In parallel with studies of human tissues, an appropriate animal model for each target site is being investigated. This approach provides a link between carcinogenesis studies using experimental animals and carcinogenesis in humans.

Model systems have been developed for human bronchus, colon, esophagus, peripheral lung, and pancreatic duct. These model systems have three major facets: 1) collection of viable human tissues; 2) in vitro maintenance of the tissues; and 3) xenotransplantation of human tissues into immunodeficient animals to assess tumorigenicity of suspected malignant cells transformed in vitro. Human tissues can be exposed to carcinogens and to anticarcinogens while in culture and/or as a xenograft. Explant cultures of human tissues have the advantage of retaining normal cellular differentiation and the 3-dimensional intercellular relationships. Monolayer epithelial cell cultures are being initiated from explants and used for carcinogenesis studies.

The long-term organ culture of human bronchus, colon, esophagus, peripheral lung and pancreatic duct was previously reported. During this fiscal year the serial passage and clonal growth of human bronchial and esophageal epithelial cells provides new opportunities for the study of carcinogenesis and differentiation in these human cells.

The culture of target epithelial tissues and cells from experimental animals and humans in a controlled experimental setting has allowed a series of interlocking studies including: 1) metabolism of chemical carcingens; 2) DNA damage by carcinogens; 3) mutagenesis in mammalian cells mediated by human tissues and cells; and 4) function, differentiation and transformation of human epithelial cells.

I. The Metabolism of Environmental Chemical Carcinogens

Studies of carcinogen metabolism were conducted in tissues from experimental animals and humans.

Cultured human bronchi, esophagus and colon were found to activate metabolically the following procarcinogens into forms that bind to cellular macromolecules including DNA: polynuclear aromatic hydrocarbons -- 7,12-dimethylbenz[a]anthracene, 3-methylcholanthrene, benzo[a]pyrene (BP), and dibenz[a,h]anthracene; N-nitrosamines -- N-nitrosodimethylamine (DMN), N-nitrosodiethylamine (DEN); hydrazine -- 1,2-dimethylhydrazine (1,2-DMH); and a mycotoxin -- aflatoxin B_1 (AFB_1).

Important differences in the metabolic activation of cyclic N-nitrosamines were found among human tissues. Human bronchus activated N-nitrosopiperidine (NPd), N-nitrosopyrrolidine (NPy), and N,N'-dinitrosopiperazine (DNP). Human colon activated NPy but binding of NPd was not found. Neither NPy nor NPd were bound to DNA in cultured human esophagus.

The metabolic pathways leading to the formation of DNA adducts in cultured bronchus, colon and esophagus have been defined for BP, DMBA, AFB_1 and DMN. The pathways to organic-extractable and water-soluble metabolites of BP were qualitatively similar in all 3 tissues; sulphate esters and glutathione conjugates were the major water-soluble metabolites.

The extrapolation of carcinogenesis data among animal species depends in part on qualitative and quantitative differences between metabolic activation and deactivation of procarcinogens. Therefore, the metabolism of BP by cultured tracheobronchial tissues from different species -- human, bovine, hamster, rat and mouse -- has been investigated. The total metabolism, as measured by both organic solvent-extractable and water-soluble metabolites of BP, was substantial in the respiratory tract from humans and from animal species susceptible to the carcinogenic action of BP. The ratio of organic-extractable metabolites to water-soluble metabolites was greater than one in hamster, human and C57Bl/6N mouse, but less than one in rat, bovine and DBA/2N mouse, suggesting that determination of both activation and deactivation pathways are important in assessing carcinogenic risk of a chemical. Sulphate esters and glutathione conjugates were the major water-soluble metabolites in all animal species; tetrols and diols were the major organic extractable metabolites. The level of trans-7,8-dihydro-7,8-dihydroxybenzo[a]pyrene, the proximate carcinogenic form of BP, was three times higher in C57Bl/6N, than in DBA/2N mouse trachea. Trans-9,10-dihydro-9,10-dihydroxybenzo[a]pyrene was the major metabolite formed by cultured hamster trachea. The binding levels of BP to cellular DNA were quite similar in all tissues, although slightly higher binding was observed in hamster trachea. Wide interindividual variation in the binding of BP to DNA was seen in tissues from outbred species. The major BP-DNA adducts in all animal species were formed by interaction of benzo[a]pyrene diol-epoxide with the 2-amino group of deoxyguanosine. Both stereoisomeric forms of (±)-(7β,8α)-dihydroxy-(9α,10α)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE I) reacted with

deoxyguanosine, the (7R)-form being the most reactive. No difference in the relative distribution of the various adducts was seen between the species except in the CD rat, where BPDE-deoxyadenosine adducts accounted for 20% of the total modification. In cultured hamster trachea the persistence of the different adducts was similar. In conclusion, the metabolism of BP is qualitatively similar in tracheobronchial tissues from both humans and animal species in which BP has been experimentally shown to be carcinogenic.

An individual's risk of developing cancer may depend, in part, on the balance between activation and deactivation of chemical procarcinogens. We have previously found wide quantitative differences (50- to 150-fold) in carcinogen bound to DNA in cultured human tissues. This wide interindividual variation is similar in magnitude to that found in pharmacogenetic studies of drugs. Whether or not these interindividual differences in carcinogen binding values reflect similar differences in oncogenic susceptibility to these procarcinogens, is an important question for investigation. For example, in a combined epidemiology-laboratory study we are comparing the metabolism of BP in cultured non-tumorous bronchial mucosa from cancer and non-cancer patients. Patient history and clinical findings are obtained from interviews and hospital records. Lung cancers are classified by cytochemistry and both light and electron microscopy. Macroscopically normal bronchial explants, collected at time of surgery or immediate autopsy, are maintained in chemically-defined medium for 7 days. BP (1.5 μM) is then added for 24 hr. Binding levels of BP to mucosal DNA (pmoles per 10 mg DNA) are being measured as part of this study. In the initial 79 patients (21 without and 58 with lung cancer), the interindividual variation among samples from single cases is less than 2-fold. When compared to binding levels in non-cancer patients (18, mean; 2-82, range) higher levels are found in bronchi from patients with (a) epidermoid differentiated carcinomas including combined epidermoid-adenocarcinoma with a well-differentiated epidermoid component (34; 2-111; $p < 0.01$) or (b) mucous differentiated cancers with a non-glandular pattern (35; 11-96; $p < 0.025$), while no significant differences are found in bronchi from patients with glandular, mucous differentiated cancers (15; 1-45). Patient age, sex and history of tobacco smoking and consumption of alcholic beverages are not confounding factors for the associations between binding levels and tumor types.

The effects of exogenous agents on the induction and the inhibition of the enzymes responsible for the activation of BP in cultured human bronchus, peripheral lung and pulmonary macrophages have been noted in previous annual reports. In general, inducing agents and inhibiting agents in experimental animals produce similar effects in these cultured human tissues and cells. During this fiscal year these studies have been extended to investigations in cultured human colon.

The binding of BP to DNA was inhibited by addition of antioxidants (butylated hydroxytoluene and disulfiram) to the media, while coincubation with either lithocholic acid (1 μM) or chenodeoxycholic acid (2.5 μM) significantly increased the binding value of BP. These bile acids caused an

enhancement of the overall BP metabolism. None of the other bile acids -- cholic acid, deoxycholic acid, taurodeoxycholic acid -- showed any persistent effect on BP binding.

In summary, human bronchus, colon and esophagus can metabolize polynuclear aromatic hydrocarbons, N-nitrosamines, hydrazines and mycotoxins that are found in the environment and/or tobacco smoke. Metabolic pathways and carcinogen-DNA adducts were generally similar among animal species including humans. Wide quantitative differences in carcinogen binding to DNA was found among different people. The relationship between this finding and an individual's oncogenic susceptibility to environmental procarcinogens requires further study. Finally, organ specificity of chemical procarcinogens may depend in part on inter-tissue differences in metabolism of the organotropic carcinogens. Quantitative and, in the case of cyclic N-nitrosamines, perhaps qualitative differences have been found among human tissues.

II. DNA Damage by Carcinogens

DNA damage is considered to be important for the oncogenic effects of many chemical carcinogens. As noted above, we have extensively studied the metabolism of chemical procarcinogens and have identified their DNA adducts in cultured human tissues and cells. The next logical step in these investigations is to determine the intra- and inter-genomic distribution of these adducts, DNA repair processes in human epithelial cells, and the biological consequences of these carcinogen-DNA interactions. Such studies are now feasible because of recent advances in molecular biology made by others and the following contributions by the HTSS and coworkers: (a) development of ultrasensitive enzymatic radioimmunoassay for the quantitative measurement of femtomole amounts of antigens including carcinogen-DNA adducts; (b) hybridoma production of monoclonal antibodies to carcinogen-DNA adducts; and (c) methods for the serial culture of human bronchial epithelial cells.

By combining radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), we have developed a very sensitive immunoassay -- ultrasensitive enzymatic radioimmunoassay (USERIA). Experiments were conducted to modify RIA or ELISA so that antigens or antibodies could be measured by the USERIA procedure, and to compare the sensitivity among these immunoassays. So far USERIA has proved to be the most sensitive immunoassay. It is 100- to 1,000-fold more sensitive than RIA in the detection of rotavirus, cytomegalovirus, and cholera toxin. The procedure was modified to determine covalent binding of chemical carcinogens to DNA. The USERIA procedure which measures benzo[a]pyrene (BP)-DNA adducts and acetylaminofluorene (AAF)-DNA adducts was 100-fold more sensitive than RIA. As few as 3 fmole AAF-DNA adducts in 10 ng DNA can be measured by non-competitive USERIA and 2 fmole in 1 μg DNA by competitive procedures. With similar procedures, 4 fmole of BP-DNA adducts in 10 ng DNA can be detected by non-competitive USERIA, and 10 fmole in 100 μg DNA ($\sim$1 molecule of BP-DNA adduct per 10^7 nucleotides) by competitive methods.

Monoclonal antibodies to carcinogen-DNA should provide investigators with highly specific reagents for both clinical and molecular studies. We have

initiated a program to produce such monoclonal antibodies. For example, mouse myeloma cells (P_3x63) were fused with spleen cells from Balb/c mice immunized with AFB_1-DNA adducts. Hybrid cells were grown in selective medium and tested for production of antibody-secreting hybridomas. Clones secreting monoclonal antibodies binding specifically to AFB_1-DNA adducts have been obtained and are being characterized.

Most studies of DNA repair in mammalian cells have been conducted in cultured fibroblasts and lymphocytic cells. With the successful culture of human bronchial cells, discussed in a later section, DNA repair is now being studied in normal human epithelial cells. Using alkaline elution methodology developed by Kohn and coworkers, we have found that human bronchial epithelial and fibroblastic cells repair DNA damage by either x-irradiation or metals, i.e., chromic compounds. These studies are being extended to asbestos and to chemical carcinogens.

As a prelude to similar studies in human epithelial cells, we have studied carcinogen-DNA interactions in Chinese hamster cells. For example, the analysis of sister chromatid exchanges (SCE) is often proposed as a sensitive and quantitative assay for genetic damage of the sort leading to mutation and/or cancer. We have found agents that produce varying types of DNA damage but an undetectable increase in mutation frequency. We asked whether any of these agents would produce SCEs, and, if so, whether any correlation could be drawn between DNA damage and SCE formation. In V-79 cells, mutation was measured as resistance to 6-thioguanine, toxicity was measured by the loss of plating efficiency, and DNA damage was measured by alkaline elution. The results show that the undetectably mutagenic agents, transPt. (II) diammine-dichloride, visible light from a GE 275_W sunlamp above 350 nm (1.8×10^5 J m^{-2}), and hydrogen peroxide produce significant increases in SCE frequency. The mutagenic agents, cis-Pt. (II) diammine-dichloride and the sunlamp at wavelengths above 305 nm (2.4×10^5 J m^{-2}), also produced large increases in SCE frequency. DNA-DNA crosslinks were produced only by the Pt. complexes, while DNA single strand breaks were produced by the other agents. DNA-protein crosslinks were produced by all agents except H_2O_2. Thus, so far no consistent correlation between various DNA lesions and SCEs has been found. We conclude that lesions leading to SCEs and certain types of DNA damage will not necessarily produce mutations.

To study the relationship of mutation, SCE, cytotoxicity and nascent DNA synthesis, V-79 cells were treated with B[a]P diol epoxide I and II. Both isomers caused dose-dependent decreases in survival of cells and in the size of nascent DNA. Increases in the frequencies of SCE and of O^r mutations were found in cells treated with either isomer. However, B[a]P diol epoxide I caused 10-20-fold more O^r mutations and 100% more SCE than did B[a]P diol epoxide II at equal molar dose levels. In contrast, the isomers induced similar reductions in the size of the nascent DNA at equal dose levels. Caffeine, at non-toxic dose levels, caused a synergistic reduction in cell survival induced by both isomers and also inhibited DNA elongation. However, caffeine did not affect the induction of either O^r mutants or SCE by either isomer. Thus the results suggest

that the reduction in the size of nascent DNA by both isomers was more closely related to cytotoxicity than to the induction of SCE or 0^r mutations.

III. Human Tissue- and Cell-Mediated Mutagenesis

Since most chemical carcinogens are also mutagens, assays of mutagenesis are useful as screening tests for chemical carcinogens. In many of these assays, promutagens (procarcinogens) are activated by microsomal preparations from either experimental animals or from human tissues. However, metabolic activation of procarcinogens by microsomal preparations may not reflect metabolic activation within intact cells. A mutagenesis assay has been developed in which promutagens are metabolically activated by explants of human target tissues, e.g., bronchus. Mutation frequency (resistance to either 8-azaguanine or ouabain) is measured in a mammalian cell line, V-79 Chinese hamster cells, which does not metabolically activate polynuclear aromatic hydrocarbons into mutagens. When human bronchial explants are cocultivated with V-79 cells in medium containing either BP or its proximate carcinogen, 7,8-diol, a marked increase in mutation frequency is observed. This increase is dependent upon doses of either BP or 7,8-diol, duration of cocultivation, and amount of human bronchus. At equimolar concentrations, 7,8-diol was activated to at least a 5-fold more potent mutagen than its parent compound, BP. A positive correlation between binding level of BP to bronchial DNA and frequency of ouabain-resistant mutations in V-79 cells was found. As noted before, pulmonary alveolar macrophages can also activate chemical carcinogens and mediate mutagenesis in co-cultivated V-79 cells. Our data are consistent with the hypothesis that macrophages and bronchial epithelium may act in concert metabolically to activate inhaled chemical carcinogens and focus attention on one facet of the possible interactions between non-target and target cells of carcinogens during carcinogenesis. Human tissue- and cell-mediated mutagenesis tests also have potential for: 1) screening tests for chemical carcinogens; 2) assays to be used in an effort to determine oncogenic susceptibility among individuals as well as among different target tissues in a single individual; and 3) studying the possible interactions between non-target and target cells in the metabolsim of chemical carcinogens.

IV. Function, Differentiation and Transformation of Human Epithelial Cells

A method for routinely initiating replicative epithelial cell cultures of human bronchus has been developed. Large pieces of bronchus tissue are initially set-up as explant cultures and incubated in a rocking chamber for two to three days to facilitate removal of mucus. These explants are then cut into smaller pieces and explanted into medium containing less than 2% serum. The low serum concentration permits rapid outgrowth of epithelium but retards growth of the fibroblastic cells. Thus, after 5-10 days incubation, very few fibroblastic cells are present among the epithelial outgrowth. At this time the tissue explant is transferred to a new dish for reseeding a second wave of epithelial cell outgrowth. The epithelial cells remaining on the dish cease dividing and eventually die unless Swiss mouse 3T3 feeder cells are added to the cultures. These feeder cell-suppor-

ted bronchial epithelial cells continue to divide rapidly. Our studies have yielded the following information: (1) epithelial cells have the normal human karyotype; (2) the human bronchial epithelial cells have been subcultured at low density and clonal growth has been accomplished. Feeder cells and 2% serum supplement were necessary for growth of the subcultured cells; (3) coating the dish surface with fibronectin and adding epidermal growth factor (EGF) and cholera toxin to the nutrient medium improves clonal plating efficiency (from 2 to 15%) and increases the clonal growth rate (2.5-fold). The addition of other factors, e.g., MSA, FGF and EGGS were less mitagenic; (4) cultures of human bronchial epithelium average 35 population doublings *in vitro*; (5) epithelial cell markers have been demonstrated. Keratin was shown by both immunospecific, Kreyberg and rhodamine B staining. Blood group antigens were also detected by immunospecific staining. However, mucopolysaccharide staining was negative. Ultrastructurally, these cells have numerous tonofilaments and desmosomes; (6) the calcium concentration of the medium influences colony morphology and cell shape. The cells are small and tightly polygonal when growing in normal (1 mM) levels of calcium. Progressive reduction of the calcium concentration to 0.06 mM causes the cells to become flatter and less closely associated; and (7) EGF decreases the number of desmosome junctions between cells. *In vitro* carcinogenesis results with long-term cultures of human bronchial tissue are: (1) explant cultures have been maintained for more than 1 year. A steady state condition has been established whereby dying cells are replaced by cells migrating from the tissue. The older cultures consist predominantly of spindle-shaped cells; (2) thirteen successive epithelial cell cultures have been initiated by repeatedly transferring a tissue fragment. After 6 months, epithelioid cells are still the prominent cell type migrating out from the tissue. However, fibroblastic cells become dominant in these cultures within two months after transfer of the tissue fragment unless feeder cells are added to the cultures. After eleven transfers, secondary cultures of carcinogen treated cells grow more rapidly with feeder cells than secondary cultures of nonexposed control cells. The exposed cells retained the normal karyotype through at least 9 transfers; (3) asbestos fibers rapidly penetrate the plasma membrane of the epithelial cells. High voltage and scanning electron microscopy studies, still in progress, have shown asbestos in the cytoplasm, and many fibers were protruding through the plasma membrane after 24 hrs incubation; (4) carcinogen treatment caused the modal chromosome number of SV40 infected human prostatic epithelial cells to increase from 46 to more than 60. These exposed cells have been xenotransplanted; and (5) the *in vivo* carcinogenesis investigations are still in progress. Polonium 210 has been implanted into the lumen of xenotransplanted subsegmented bronchi.

V. Other Activities

This section has been responsible for training intramural and extramural investigators in the techniques for culturing human tissues and cells. In addition, two NIH Conferences entitled "Methods to Culture Normal Human Cells and Tissues" and "Variation in Cancer Susceptibility" were organized, and the published proceedings of these Conferences edited by the staff of this section. Members of our staff have also served on the

ad hoc Chemical Pathology Study Section, Pathology B; and served as consultants and/or committee members for the Gene-Toxicology Program (EPA), SCORE Program (NHLBI), Carcinogenesis Risk Assessment Committee (FDA), Environmental Pathology Registry Committee (AFIP), and Cellular Aging Research (NIA).

IN VITRO PATHOGENESIS SECTION: "(1) Develops in vitro models for studying the pathogenesis of major forms of human cancer utilizing epithelium from both human and animal tissues; (2) utilizes these culture systems in biochemical, cytological and biological studies to investigate the mechanism of carcinogenesis and to develop approaches to the prevention of cancer; (3) studies the stages in the pathogenesis of cancer to identify markers for preneoplastic lesions so that such lesions can be arrested or reversed prior to their becoming a threat to life; (4) develops procedures for evaluating the biological potential of lesions induced by chemical or physical carcinogens in mammalian tissues in vitro".

This section has directed its efforts toward both developing in vitro model systems to study chemical carcinogenesis in epithelial cells and to use these systems to study the mechanisms of tumor initiation and promotion. The classic model for induction of squamous cancer by chemicals, mouse epidermis, has been adapted for in vitro study. Previous investigations had demonstrated that this model is a close in vitro analogue of the mouse skin carcinogenesis system in vivo. In vitro, epidermal cells proliferate and differentiate, metabolize carcinogens, repair DNA damage, and respond to tumor promoters like epidermis in vivo.

Regulation of Epidermal Growth and Differentiation: During the past year, both our understanding of the fundamental biology of the mouse epidermal culture system and the usefulness of this model for carcinogenesis studies have been greatly enhanced. The recent discovery that ionic calcium is a critical regulator of epidermal growth and differentiation have provided a method to control and study each of these phases of the keratinocyte life cycle. At low ionic calcium concentrations in culture medium (0.02 - 0.09 mM), epidermal cells maintain a monolayer growth pattern with a high proliferation rate. Essentially 100% of the attached cells are in the proliferating pool and these cells have morphological, enzymological and immunological characteristics of basal cells.

When cells maintained under low calcium growth conditions are switched to medium with calcium contents above 0.1 mM (standard culture media are 1.2 - 1.8 mM), differentiation is induced. The earliest changes occur within a few minutes. During a 72 - 96 hour period, the cultures undergo vertical stratification, form keratohyalin granules and cornified envelopes, and mature sheets of squamous cells are sloughed into the medium. DNA synthesis decreases rapidly and is essentially stopped by 36 hours after calcium addition while RNA and protein synthesis decrease more gradually.

The mechanism by which calcium induces differentiation in epidermis appears to be specific since many other cations, both organic and inorganic, failed to reproduce the effect. Inhibitors of macromolecular synthesis, microtubule disrupting drugs and agents which interfere with intracellular calcium function were without significant effect. Several drugs which influence the membrane transport of calcium were effective in inhibiting calcium induced differentiation. These results suggested that membrane charge potential, membrane ion flux or other cell surface effects, rather than elevation of intracellular calcium, are responsible for the induction of differentiation.

Quantitative Assay for Carcinogen-induced Altered Differentiation: The regulation of epidermal proliferation and differentiation by calcium forms the basis of a new in vitro assay developed for detecting carcinogen induced changes. Reasoning that during the process of carcinogenesis an alteration in differentiation must occur, epidermal cells were grown in low calcium medium and treated with carcinogens. Colonies which were resistant to calcium-induced cell death by terminal differentiation were then selected by raising the calcium levels in culture medium. Selected colonies were fixed and stained with rhodamine, a stain for keratinizing colonies. To date, results of studies with two carcinogens N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz[a]anthracene (DMBA) are available. In 5 experiments, representing a total of 319 cultures, the number of continuously growing colonies selected from carcinogen-treated cultures exceeded that in controls (spontaneous variants) by as much as 30 fold with an average of 8 fold. Dose-response studies with both MNNG and DMBA reveal an increase in transformation frequency with dose of carcinogen when correction for toxicity is made. Reproducibility within an experiment is excellent for the same dose of carcinogen. Colonies derived in this way are generally discreet and quantifiable. All colonies are composed of epithelial cells with characteristic epidermal markers. Such cells do not grow in agar and have not produced tumors in animals within 6 months after subcutaneous injection into syngeneic newborns. Thus this assay may select for preneoplastic cells which arise spontaneously in epidermal cell cultures or which can be induced by carcinogens. Alternatively, these changes may be unrelated to the process of carcinogenesis. Studies are in progress to determine if cells selected in this manner progresss to the fully malignant stage either spontaneously or by further treatment with carcinogens or tumor promoters. Conditions for the clonal growth of epidermal cells have been defined recently, and subclones of keratinocytes with high cloning efficiencies have been isolated. Such clones are presently being used to establish a clonal assay for carcinogen induced altered differentiation.

Phenotypic Markers for Malignant Epidermal Cells: The identification of markers for transformed epidermal cells has utilized cell lines which yield squamous cell carcinomas upon injection into syngeneic mice. Only 1 of 12 lines grew in agar, and the use of agarose or methocel failed to improve anchorage-independent growth. All lines studied retained differentiative characteristics including the synthesis of keratin proteins, near normal protein patterns by gel electrophoresis and the production of pemphigus, pemphigoid and stratum corneum basic protein antigens. Endogenous activity of the enzyme ornithine decarboxylase, reported to be elevated in epidermal carcinomas in vivo, was variable in these lines as was the induced level after exposure to TPA. The only consistent alteration noted was the behavior of all lines to changes in medium calcium concentrations. Whereas low calcium conditions resulted in identical monolayer behavior as seen in normal epithelial cells, a switch to high calcium failed to induce cell death associated with terminal differentiation. Thus a defect in calcium-induced differentiation, similar to that employed to select carcinogen treated primary cultures (thought to be preneoplastic variants), was reproduced in these malignant

lines. When a small number of malignant cells was mixed with a larger population of normal cells and grown under low calcium conditions, selection in high calcium resulted in colonies derived from the malignant cells whereas controls (with no added transformed cells) failed to yield colonies. This provides support for the use of calcium selection as a basis for a transformation assay in epidermal cell culture.

Human Epidermal Cell Culture and Specific Assays for Differentiation Products: To extend our findings from mouse epidermal cells to human tissues, we have established conditions for long term maintenance of human keratinocytes in culture. By the use of reduced calcium concentration, collagen substrate and controlled culture conditions, neonatal foreskins can be maintained in vitro for 4 - 5 months and undergo up to 9 subcultures. During this time the cells preserve their functional integrity as determined by the synthesis of a variety of epidermal specific proteins. Carcinogenesis studies are currently in progress with this new culture model. To enhance our capability to study epidermal function, a radioimmunoassay has been established for epidermal keratin. This assay, the most sensitive technique available for this purpose, can detect picomoles of keratin proteins in cell and tissue extracts. Although mouse keratin is detected with greatest efficiency, hamster, bovine, rat, and human keratin can also be quantified. Using this assay, keratins have been detected in a variety of mouse tissues other than skin and in human epithelial cell cultures.

Determinants for Susceptibility to Carcinogenesis: Studies to understand the mechanism of tumor initiation have attempted to determine the basis for strain susceptibility to skin carcinogenesis and have also focused on the interactions of carcinogens with DNA. Epithelial cells from Sencar strain mice, which are highly susceptible to two-stage skin carcinogenesis, have been studied in culture. When grown under low calcium conditions for 6 weeks without treatment and selected for resistance to differentiation induced cell death, 13 fold more colonies were observed in Sencar cultures compared to Balb/c cultures. Likewise when Sencar cells were cloned as primary cultures, colony size at 2 weeks was 10 fold greater than Balb/c cells cloned at the same time. These results suggest that Sencar mice have inherent changes in the biological behavior of their keratinocytes, and such changes may be associated with the increased susceptibility of these cells to chemical carcinogenesis.

Radioimmunoassay for Carcinogen-DNA Adducts: The interaction of carcinogens with DNA has been studied by a unique methodology pioneered by this Section. Antibodies have been developed in rabbits against N-(8)-guanyl-2-acetylaminofluorene (G-8-AAF) and N-(8)-guanyl-2-aminofluorene (G-8-AF) the major guanosine adducts formed in vivo and in vitro by the interaction of nucleic acids with the aromatic amine carcinogen 2-acetylaminofluorene (AAF). The antisera obtained have been utilized to detect picomoles of each C-8 adduct in a competition radioimmunoassay utilizing ^{3}H-G-8-AAF or ^{3}H-G-8-AF. A linear regression equation has been established by computer techniques which allows precise quantitation of each C-8 adduct in an unknown mixture in which dG-8-AAF comprises less than 10% of adduct; for mixtures with more than 10% dG-8-AAF, proportions can be estimated

by comparison with standard mixtures. A variety of cultured cell types exposed to N-acetoxyacetylaminofluorene have been surveyed to determine the innate capacity of each type to deacetylate the carcinogen during the process of adduct formation. The C-8 adduct in most cell types studied (mouse and rat keratinocytes and fibroblasts, human fibroblasts) was >90% deacetylated (dG-8-AF). Rat hepatocytes in primary culture were exceptional in that they contained >80% of adduct as acetylated dG-8-AAF. The acetylated form was also the major product when cultured hepatocytes were exposed to AAF. However, DNA from livers of rats fed or injected with AAF contains a sizable portion of the C-8 adduct as deacetylated, suggesting that cultured hepatocytes may not be performing the same metabolic steps as rat liver in vivo.

In mouse keratinocytes cultures, the pattern of binding can be shifted to 100% dG-8-AAF and the level of binding markedly reduced by exposing cells to paraoxon, a microsomal deacetylase inhibitor, just prior to carcinogen exposure. This provides a way to manipulate the adducts formed in order to determine the relative carcinogenicity of each adduct. Antisera have also been developed against DNA substituted with the 7,8-diol, 9,10 epoxide of benzo[a]pyrene (BPDE I, the anti-isomer), and these antisera show specificity to the trans(7R)-(benzo[a]pyrene)-N^2-deoxyguanosine (BPdG) adduct as well as to BP substituted native and denatured DNA. Since >90% of the BP adducts in epidermal cells in vivo or in vitro are G adducts as determined by high pressure liquid chromatography, the RIA established with these sera is a valid measure of most of the DNA binding produced by BP and derivatives. During 24 hours after exposure to BPDE, approximately 40% of the guanosine adducts are removed as detected by RIA. Indirect immunofluorescence of epidermal cells exposed to BPDE yields intense nuclear fluorescence when the BPdG antiserum is used, but no fluorescence with preimmune serum.

Mechanisms of Action of Tumor Promoters and Antipromoters: Tumor promotion by phorbol esters has been an area of intense study in this laboratory. In the last year we have focused on the events associated with the initial responses of epidermal cells exposed to phorbol esters and particularly 12-O-tetradecanoylphorbol-13-acetate (TPA). Other studies are directed toward understanding the effect of this interaction on cellular function and the mechanism by which antipromoters interfere in this process. Phorbol ester induced increase in activity of the enzyme ornithine decarboxylase (ODC) has been a reliable early marker for promoter effects in epidermis. While it is known that this induction requires transcription and translation, the mechanism of ODC gene activation is not understood. For this reason, agents which induce more defined genomic alterations have been studied for their effect on ODC induction alone and in concert with TPA. Ultraviolet light (10 J/m^2) (UVL) induces ODC with biphasic kinetics and peak inductions at 4 hr and 15 hr after exposure. At lower doses (2-5 J/m^2) UVL enhances ODC induction by TPA and at higher doses (20 J/m^2) it suppresses TPA-induced ODC. The inducing and enhancing properties of UVL can be separated by appropriate culture conditions. Mitomycin C produces DNA strand breaks and DNA-protein crosslinks. It induces ODC in a biphasic pattern similar to UVL but has no enhancing

properties on TPA induction. Hydrogen peroxide damages DNA by a free radical mechanism and induces ODC with a peak at 4 hrs after exposure. It does not enhance TPA induction. Thus DNA damaging agents induce the enzyme in a manner similar to UVL but do not have the enhancing properties of UVL. Whether DNA damage at the site of the ODC gene is critical for its activation and whether TPA can produce this damage remains to be determined. The availability of inducers of ODC with partially distinct mechanisms of action (UVL and TPA) has provided a way to study the site of action of a variety of agents which alter the induction or alter tumor promotion. Our data suggested that the early portion of the induction pathways differ for UVL and TPA while the transcription and translation portions are probably common. Retinoids are more potent inhibitors of TPA induction than UVL induction and thus probably inhibit an early event. Local anesthetics and the protease inhibitor TLCK inhibit both inductions equally and probably act on the common pathway. Local anesthetics appear to act in a similar way to polyamines, probably by inhibition of enzyme translation.

The steroid fluocinolone acetonide enhances both activities to the same extent and probably acts on the common pathway also, possibly by stabilizing the ODC messenger RNA. Epidermal differentiation appears to be associated with a loss of TPA responsiveness. Under low calcium growth conditions (basal cells) TPA responsiveness is greatly enhanced and sustained for prolonged culture periods. Shortly after induction of differentiation by calcium, TPA no longer induces ODC. Other changes are noted after TPA exposure to cells in low calcium. The enzyme epidermal transglutaminase is induced and the secretion of a 35K glycoprotein is greatly enhanced. The synthesis of a number of new proteins is initiated by TPA exposure as determined by two-dimentional gel electrophoresis. Thus TPA appears to activate a program of cellular responses in epidermal cells. Which of these responses is critical for promotion must still be determined.

PERINATAL CARCINOGENESIS SECTION: "(1) Investigates the induction of cancer in experimental animals before birth and during infancy, with the goal of identifying factors responsible for the initiation and growth of tumors of childhood and infancy in man; (2) utilizes techniques of chemical synthesis, biochemistry, histopathology, immunology, and endocrinology to identify carcinogens to which both individually and in combination the fetus and neonate are particularly vulnerable, and to identify host factors which qualitatively modify their biological effects; and (3) develops measures for prevention of cancer in children or in later life as a response to conditions of high susceptibility to carcinogens during fetal life or childhood."

The program of this section continues to have two major components. A. Tumor induction experiments in animals. These studies seek to expand the limited data base now available from which to define the nature of prenatal risk to carcinogens and its relation to the major neoplastic diseases of man. Their purpose is to identify characteristic changes in susceptibility to chemical carcinogenesis as the stages of prenatal life progress from blastocyst to embryo to fetus, and after birth as neonates progress to juveniles and then mature to adults. Qualitatively, these studies seek to characterize (1) differences in the spectrum of organ systems affected by a given class of chemical carcinogens as development progresses; (2) how these spectra may vary from one species to another; (3) and, within a given organ system, how specific tumor types may arise depending on the stage of differentiation and development at which carcinogen exposure occurs. Quantitatively, dose/response relationships in the induction of tumors and related lesions are investigated for selected classes of chemical carcinogens during different stages of prenatal and postnatal development. These data contribute to the estimation of relative human risk during prenatal and postnatal life. Studies of this sort require contract-supported resources for the housing and maintenance of animals, for their treatment, and for collection and histologic processing of normal and diseased tissues. B. Studies on mechanisms related to prenatal susceptibility to carcinogenesis. In order to investigate and interpret the physiologic bases for susceptibility differences and to provide a rational basis for extrapolation to man, studies are in progress on several factors which appear to play major roles in determining age dependent, organ specific susceptibility to major classes of genotoxic chemical carcinogens. These include (1) the role of cellular proliferation, and variation in susceptibility to neoplastic transformation during specific stages of the cell cycle; (2) variations in capacity to repair damage to DNA in various organ systems and in different species during development; (3) the role of cocarcinogenic agents in promoting the phenotypic expression of neoplastic transformation, presumably consequent to carcinogen-initiated changes in the genome of target cells; (4) the relative roles of maternal and fetal metabolism in determining the organotropism and quantitative effects of chemical carcinogens of different classes in fetal and adult rodents; and, most uniquely relevant to transplacental carcinogenesis; (5) the role of state of differentiation and of its controlling factors, including both exogenous substances and cell-cell interactions, in controlling the expression of neoplastic transformation in selected organ systems, especially the kidney. In order to accomplish these studies, which cover a wide range of specialties and areas of expertise, it has been necessary to establish collaboration with several groups of investigators both within and outside NIH, including laboratories in foreign countries. Studies on the relation of specific stages of

the cell cycle to carcinogenic susceptibility have been carried out in collaboration with colleagues at the University of North Carolina School of Medicine; investigations on transplacental carcinogenesis in primates, with the NINCDS and the University of California at Davis; and studies on the biology of neoplastic liver cells in rats and mice, with investigators at the American Health Foundation, Valhalla, New York. In addition, this Section has collaborated with the Office of the Chief of this Laboratory (see its report) in research on combined effects of exposures to multiple carcinogens in mutagenesis and cell transformation.

Previous reports have documented indirect studies in this Section on the roles of maternal and fetal metabolism in determining fetal susceptibility to transplacental carcinogens in rodents and primates by carcinogenesis experiments involving agents selected from the following three classes of chemical carcinogens: agents which act directly without metabolic processing; metabolism-dependent agents with short-lived chemical intermediates including nitrosamines; and metabolism-dependent agents with long-lived metabolic intermediates, such as the polynuclear aromatic hydrocarbons.

Carcinogenesis studies in primates. During the past year, studies on a direct acting alkylating agent, ethylnitrosourea (ENU), have been continued in the Old World (African) monkey, Erythrocebus patas, and have been extended to an unrelated Asian species, the rhesus (Macaca mulatta). This year for the first time, primary tumors of the nervous system have been observed in patas monkeys exposed to ENU during gestation, extending to primates the observation from rodent studies that the nervous system is especially susceptible to carcinogens during prenatal development. Previous results suggesting that the earlier stages of prenatal development in primates are exceptionally susceptible to carcinogenesis have been confirmed by additional new data. Another new finding of major significance during the past year is that tumors of lining epithelia, especially of the lower intestinal tract, have appeared in the offspring of patas monkeys given ENU during pregnancy. The importance of intestinal neoplasia in human cancer emphasizes the significance of this finding that tumors of the intestinal mucosa, so commonly a disease of maturity in man, can result from transient exposure of primates to a carcinogen at a very early and highly susceptible stage of development during prenatal life.

Transplacental carcinogenicity studies with ENU in the rhesus monkey have revealed that, like the patas, offspring of animals given ENU during the early stages of gestation developed lethal tumors, especially of connective tissues and including sarcomas of the lung and lymphoreticular neoplasia. All neoplasms detected so far have arisen during the first year of postnatal life. No tumors have been seen in rhesus monkeys exposed to comparable doses of the carcinogen as adults. This result confirms the significance of previous studies in the patas indicating the quantitatively greater susceptibility of primates to carcinogens during prenatal life, and demonstrates that results previously obtained in the patas are not unique to that species, but are applicable to other species of subhuman primates and, with high probability, must be considered predictive for man.

Organ culture studies on renal differentiation. Improvements have been made in the organ culture system previously developed for the in vitro differentiation of kidney rudiments from mice and rats, in order that renal differentiation and its relation to the expression of the neoplastic phenotype may be systematically investigated. It was shown that in this organ culture system both kidney rudiments from 15 day fetuses and primary undifferentiated renal tumors induced in newborn rats would grow for a period of nearly 20 days and, in the case of kidney rudiments and at least one of the tumors, would differentiate. Primary undifferentiated blastemal cell renal neoplasms have now been established as serially transplantable tumors in the F344 rat, and have been shown to be adaptable to maintenance in organ culture also. Attempts are now under way to manipulate and induce the differentiation of such tumors, in order to understand the interspecies differences in morphology and patterns of growth of perinatally induced renal tumors, by comparing the undifferentiated tumors regularly inducible in the rat with the purely epithelial renal tumors of adult type induced in mice by similar treatments.

Cell cycle and susceptibility to carcinogenesis. The role of specific stages in the cell cycle, and of cell replication, in enhancing susceptibility to carcinogenesis has been studied by refining existing approaches to inducing cell synchrony during the regenerative hyperplasia which follows partial hepatectomy in the rat. It has been found that the wave of hepatocyte proliferation that follows removal of two-thirds of the liver in 100 gram rats can be delayed up to 10 hours, and synchrony improved, by repeated intraperitoneal administration of hydrocortisone hemisuccinate at 5 hour intervals prior to the onset of DNA synthesis. DNA synthesis is monitored by incorporation of tritiated thymidine into the nuclei of residual liver cells. A single dose of the direct acting alkylating agent, methylnitrosourea (MNU), was given via the portal vein at specific times after partial hepatectomy and the process of liver regeneration by progression of residual hepatocytes through the cell cycle was established by autoradiography and microscopic determination of mitotic index. Liver cell tumors were induced most frequently at times when relatively high populations of hepatocytes were synthesizing DNA. When the proportion of hepatocytes in S was shifted in time by hydrocortisone administration, the period of increased vulnerability to hepatocarcinogenesis was shifted by a comparable interval. This confirms previous rather fragmentary data that had suggested that the S phase of DNA synthesis is a fraction of the cell cycle during which susceptibility to chemical carcinogenesis is increased. In the studies with MNU, however, a very high incidence of lethal tumors was induced in extrahepatic organs (nervous system, kidneys, intestines, odontogenic tissues, Zymbal's gland, and peritesticular mesothelium) while a relatively small fraction of the animals developed tumors in the liver, and multiplicities of liver tumors were low even at the periods of greatest susceptibility. Methyl(acetoxymethyl)nitrosamine (DMN-OAc) was therefore adopted as the carcinogen for continuation of these studies. DMN-OAc, studied in detail previously as a model compound, is a carrier form of the presumed reactive metabolite of dimethylnitrosamine. The cell cycle experiment was repeated using DMN-OAc followed by prolonged dietary administration of phenobarbital as a promoter of liver tumor development. This protocol has resulted in the appearance of very high multiplicities of tumors in the rat liver after a single exposure to the carcinogen, and a virtually negligible incidence of tumors in other organ systems. This experiment is being concluded at the present time.

Role of gamma-glutamyl transpeptidase (GGT) in carcinogenesis. Previous studies identified the possible role of the inducible enzyme GGT in determining organ specificity for carcinogenesis by modulating active transport of certain classes of chemical carcinogens including the alpha-amino acid, azaserine. Such studies have been extended to other compounds. The mechanism of the association between high GGT levels and susceptibility to toxicity from azaserine in cultured cell lines has been further explored. Not all carcinogenic or toxic amino acids are related in the same fashion as azaserine to GGT content in target cells, and it now appears that simple functioning of the enzyme for transport of the carcinogen across cell membranes is not the entire answer to this complex question. In particular, neither ethionine nor the gamma-glutamyl derivatives of certain mushroom toxins, 1,1-disubstituted hydrazines, are comparable to azaserine in their patterns of toxicity in a battery of cell lines differing in GGT content. Alternative explanations for the role of GGT in organ specificity of azaserine toxicity and carcinogenicity are being tested.

DNA damage and repair in prenatal carcinogenesis. Far reaching conclusions on the role of DNA repair in determining susceptibility to prenatal carcinogenesis in various organ systems, especially the liver and brain in rats and mice, have been based by others on published observations that tumors in the rat liver are never induced by alkylating agents administered transplacentally. The demonstration reported above that phenobarbital is a potent promoter of the development of latent neoplastic liver cells into tumors suggests a method for further testing the hypothesis that the fetal rat liver, although it sustains damage from transplacental carcinogens, repairs that damage effectively and eliminates damage from hepatocyte genomes that would otherwise result in transformation to the neoplastic phenotype. Studies to test an alternative hypothesis have been designed and are now underway. Many transformed cells may in fact be induced transplacentally in the rat liver by exposure to alkylating agents, but in the absence of cocarcinogens administered postnatally their probability of proliferating to generate tumors may be neglegible. In the event that a high incidence of liver cell tumors is demonstrated in the livers of rats given transplacental ENU followed by postnatal phenobarbital, it will require a major reevaluation of current thinking on the role of repair processes as determinants of organ specific carcinogenesis.

Alkaline sucrose gradient studies were previously reported on excision repair of damaged DNA and the effects of alkylating agents in cells cultured from fetal livers and brains of rats and mice. These studies had demonstrated a significant lack of repair capacity in the fetal rat brain in comparison with the mouse brain or the liver of either species. More recent studies by the alkaline elution procedure utilizing tissues obtained directly from rodent fetuses confirmed the overall pattern observed in the previous studies. While these results are satisfying in view of the much greater susceptibility of the rat to carcinogenesis in the developing nervous system, they fail to explain why liver cell tumors are induced in the mouse in high multiplicity while none are ever seen in response to comparable treatment in the rat. The studies with phenobarbital now under way may resolve this problem. Meanwhile experiments are continuing in order to establish the relative efficiency of excision repair in different organ systems in both rats and mice at different stages of development, following prenatal exposure to alkylating agents and to

other toxic and carcinogenic compounds. The high resolution alkaline elution technique using ethidium bromide fluorescence for DNA quantitation appears well adapted to this purpose.

A cell free system in which functional beta-galactosidase is synthesized in vitro from bacterial lac operon DNA obtained from a transducing temperate bacteriophage has been used to study the molecular basis for the differential effects of alkylating versus carbamylating activities of nitrosourea compounds, and of the striking differences within the homologous series of simple short chain alkylating agents. Previous studies with MNU and ENU have been brought to a conclusion, and it has been demonstrated that while the carbamylation reaction which is responsible for much of the toxicity of both agents can be demonstrated in the in vitro system, this reaction is more important for components of the synthetic mixture (and presumably intracellular constituents) other than DNA, and that genetic damage resulting from exposure to these agents is entirely a consequence of alkylation. While this system with its defined gene product is potentially attractive for further studies of the interaction of genotoxic mutagens and carcinogens with DNA and the consequences of such interaction, this aspect of the Section's program is being terminated in this fiscal year, because of limited resources.

Applications of research in perinatal carcinogenesis. The relevance of perinatal carcinogenesis to the programs of national and international organizations, especially in the fields of disease prevention and safety evaluation, has continued to result in frequent requests for participation in formal meetings organized to discuss the utilization of research findings in this field by regulatory agencies and others concerned with the evaluation and application of toxicologic investigations. During the past year, such activities have included participation in international biomedical cooperative agreements with European nations, including the USSR, sponsored by NIEHS; presentation of a paper at the NIOSH meeting on vinyl chloride (Bethesda, February 1980); providing advice to the Bureau of Biologics, Food and Drug Administration, on the significance of studies on carcinogenicity of vaccine adjuvants included in pediatric vaccination products; and participation in the organization of a meeting sponsored by the U. S. Environmental Protection Agency on organ specificity and interspecies extrapolation in carcinogenesis, planned for the winter of 1980-81.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04484-04-LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Study of the Effects of Retinoids on the Cell Surface of Cultured Transformed Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

None

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	3	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cell surface glycopeptides from retinoid-treated more adhesive and control mouse fibroblasts 3T12 cells were analyzed. The biosynthesis of a major cell surface glycopeptide I of approximate MW 2100 was shown to be enhanced specifically by retinoic acid. This glycopeptide was characterized as belonging to the "complex" type by treatment with exo and endoglycosidases and by chromatography on Con A Sepharose. Glycopeptide I was a minor component of the total cellular glycopeptides which contained mostly glycopeptide II of approximate molecular weight 1500. This glycopeptide was shown to be of the "polymannose" type. Retinoic acid also increased the number of receptor sites for epidermal growth factor (EGF) by six to ten fold. This effect was specific for retinoids with biological activity in other systems. Inasmuch as EGF-receptors are glycoproteins, it is suggested that the effect of retinoic acid on 3T12 cells may well be the result of its action on cell surface glycoproteins.

Objectives: To study the molecular mechanisms by which retinoids increase the adhesive properties of spontaneously-transformed mouse fibroblasts (Balb/c 3T12-3 cells) in culture; in particular to characterize cell surface glycoproteins which may be responsible for the vitamin A-induced enhanced adhesion, and to understand mechanisms of growth control.

Methods Employed:

Culturing of cells. Balb/c 3T12-3 mouse fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and cultured in 150mm diameter dishes (Falcon Plastics, Oxnard, CA) in 15 ml medium. Dulbecco's modified Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, NY) was supplemented with 10% calf serum (Flow Laboratories, Inc., Rockville, MD), 25 mM HEPES (pH 7.3), and 50 µg gentamicin/ml (Microbiological Associates, Inc., Walkersville, MD). The medium was changed daily. The initial inoculum was of 10,000 cells/cm^2 of the growth area. Retinoic acid (10 µg/ml or 3.3×10^{-5}M) in ethanol (final conc. 0.5%) or ethanol alone was added 24 hours after seeding.

Radioactive monosaccharides were dissolved in the culture medium at 10 µCi/ml and added to the cells after 24 and 72 hours of exposure to retinoic acid. Cells were used after 8 hours of exposure to the label.

Preparation of labeled glycopeptides. Total cellular glycopeptides were obtained from cells which had been exposed to [2-^{3}H]mannose for 8 hours at 32 as well as 80 hours of treatment with retinoic acid. The culture medium was removed, cells were rinsed twice with 5 ml of PBS buffer at 4°C. Cells were removed from the culture surface with a rubber policeman and collected by low speed centrifugation (800 x g for 5 minutes). The pellet was extracted 3 times with each of the following solvents: chloroform/methanol (2/1); water; chloroform/methanol/water (1/1/0.3) and the dry residue was treated with pronase in 0.1 M Tris-HCl buffer, pH 7.9 containing 10 mM $CaCl_2$ and 100 µg/ml of pronase CB (B Grade, Calbiochem, San Diego, CA). Proteolysis was carried out for 3 days with pronase (100 µg/ml), added at 0, 24 and 48 hours. A few drops of toluene prevented bacterial contamination. Pronase was then inactivated by boiling and the mixture was centrifuged to remove undigested material which contained only 7% of the total radioactivity. The supernatant was then used for analysis. Cells were incubated with 5 ml/dish (15 cm diameter) of PBS buffer, pH 7.9 containing 100 µg/ml of pronase for 5 minutes at room temperature. The fluid was then removed and cells were washed with 5 ml of PBS buffer, pH 7.9 and the fluids were combined, centrifuged at low speed and the supernatant was used for analysis. Fewer than 1 % of the cells were detached by this procedure and were collected in the pellet. Cells attached to the dish were subjected to trypan blue exclusion test, showing no change in viability by this criterion. Less than 1% of the cells took up the dye. Cells from parallel dishes were scraped with a rubber policeman in 5 ml of PBS buffer, pelleted by low speed centrifugation, and extracted three

times with each of the following solvents: chloroform/ methanol (2/1); water; chloroform/methanol/water (1/1/0.3). The residue was solubilized in 0.2 N NaOH for 2 hours at 50°C, neutralized with 0.2 N HCl and radioactivity was measured to determine the amounts of radioactivity associated with the cells. Material released by short pronase treatment of 3T12 cells was then subjected to the same extensive proteolysis as for the total cellular material.

Sephadex G50 superfine chromatography. Column (1.5 x 104 cm) of Sephadex G50 superfine (Pharmacia Fine Chemicals AB Uppsala, Sweden) was packed and equilibrated in PBS buffer, pH 7.3. Flow rate was about 8 ml per hour and fractions of 1.3 ml were collected. An equal amount of radioactivity was applied to the column for retinoid-treated and control cells. To estimate the sizes of the labeled material the sample to be analyzed was mixed with oligosaccharide chains (kindly donated by Dr. Vincent Hascall, NIH, Bethesda, MD.) of known length generated by enzymatic cleavage of standard hyaluronic acid. HA_{16}, HA_{12}, HA_{10}, HA_8, HA_4 refer to hexadeca-, dodeca-, deca-, octa- and tetrasaccharide-containing polymers of the repeating unit glucuronic acid-N-acetylglucosamine. Mannose and blue dextran were also included. Radioactivity was measured by liquid scintillation counting in a Beckman LS 250 Liquid Scintillation Counter.

Glycosidase treatments. α-Mannosidase (a gift from Dr. Gilbert Ashwell, NIH, Bethesda, MD) was obtained as a precipitate from ammonium sulphate. The precipitate was stored at 4°C. An aliquot was pelleted, dissolved in water and the activity was tested against paranitrophenol α-D-mannoside. Incubation was carried out in 50 mM sodium acetate buffer, pH 5.0; 0.2 mM $ZnSO_4$; 0.1 M NaCl; 0.1% bovine serum albumin and 10 µl of the enzyme (1.3 Units). Incubation (final volume 400 µl) was run for 23 hours at 37°C with the enzyme (1.3 Units) added at 0,6,18 hours. For these and subsequent incubations, 1 drop of toluene was added and the reactions were stopped by boiling for 3 minutes. The material was then analyzed by gel filtration.

Endoglycosidase D from Diplococcus pneumoniae and Endoglycosidase H from Streptomyces griseus were obtained from Seikagaku Kogyo Co., Tokyo, Japan, which also furnished α-L-fucosidase and β-N-acetylglucosaminidase both from Charonia lampas. For endoglucosaminidases, incubations were carried out in 0.05 M citrate-phosphate buffer, pH 5.0 for Endo-H and pH 6.0 for Endo-D with 25 mU of the enzyme (25 µl) resuspended in 0.1 M NaCl containing 0.1% BSA; final volume was 150 µl. Controls without enzyme were also run. Incubations proceeded for 15 hours at 37°C in the presence of a small amount of toluene to prevent bacterial contamination.

α-L-Fucosidase digestion was carried out in 0.05 M citrate-phosphate buffer, pH 4.0; 80 mU of the enzyme in a final volume of 450 l, at 37°C for 18 hours.

Neuraminidase from Clostridium perfringens was obtained from Worthington Biochemical Company, Freehold, NJ. Incubations were carried out in 0.05 M citrate phosphate buffer, pH 6.0 and 25 mU of enzyme in a final volume of 300 l for 1 hr at 37°C.

Concanavalin A-sepharose chromatography. Con A-Sepharose (Pharmacia Fine Chemicals, AB Uppsala, Sweden) columns (1 x 3.5 cm) were packed and equilibrated with 0.1 M sodium acetate pH 7.1, 1 M NaCl, 1 mM $MgCl_2$, $MnCl_2$ and $CaCl_2$. Fractions of 2 ml were collected and radioactivity measured as usual. α-Methyl mannoside was included in the eluting buffer at a concentration of 0.2M.

Gas liquid chromatography analysis of radioactive monosaccharides. [5,6-^{3}H]Fucose-labeled glycopeptides were hydrolyzed in 0.3 N HCl at 100°C for 1 hour and [2-^{3}H]mannose-labeled glycopeptides were hydrolyzed in 3 N HCl at 100°C for 3 hours, both in sealed ampoule under nitrogen. Other procedures for reduction of mannose to mannitol and derivatization to the boronic acid derivatives for analysis on gas liquid chromatography were as described elsewhere. The temperature for the analysis of fucose was 160°C, whereas for mannose it was 200°C. 3% OV-17 on gas chrome Q columns were used. Fucose was derivatized without reduction and fucosyl-boronate was eluted at 3 minutes at 160°C. Only radioactive mannose and fucose was found in the [2-^{3}H]mannose-labeled and [5,6-^{3}H]fucose-labeled glycopeptides, respectively.

Major Findings:

Total cellular [2-^{3}H]mannose-labeled glycopeptides from retinoid-treated and control spontaneously-transformed mouse fibroblasts (Balb/c 3T12-3 cells). Treatment of 3T12 cells with RA caused an increase in the incorporation of [2-^{3}H]mannose into lipid phase material as well as into total glyco-conjugates in the insoluble residue.

Proteolysis of the insoluble residue obtained from [2-^{3}H]mannose-labeled cells after extraction of lipids yielded a major peak of radioactivity at approximately Mr 1,500 (fraction II) from both retinoid-treated and control 3T12 cells. At this time of treatment with retinoic acid (80 hours), the cells were very adhesive (5% of cells are now detached by EDTA adhesion assay) compared to their untreated controls (90% of cells were detached). In addition to fraction II, a shoulder of radioactivity at approximately Mr 2,100 (fraction I) was also eluted. This fraction was resistant to treatment with α-mannosidase, which cleaved fraction II almost completely, yielding [^{3}H]mannose as the major product of this enzymatic hydrolysis. No differences were found between glycoconjugates from retinoid-treated and control cells both in molecular weight as well as susceptibility to α-mannosidase.

Cell surface glycopeptides: Studies on cell surface glycoconjugates showed an increase in the higher molecular weight glycopeptides (fraction I) of approximately Mr 2,100 and a corresponding decrease in the lower molecular weight fraction, fraction II as a result of treatment with RA.

Fraction I, labeled with mannose and fucose, was shown to be of the "complex" type from its susceptibility to neuraminidase, α-L-fucosidase, and Endo-D in conjunction with fucosidase, neuraminidase β-galactosidase and -N-acetylglucosaminidase, and its resistance to α-mannosidase and Endo-H. This fraction was a minor component of the total cellular glycopeptides, whereas it constituted a major component of the cell surface glycopeptides. Neuraminidase treatment of cell surface glycopeptides yielded the same size glycopeptide (about Mr 1,800) from fraction I as treatment with Endo-D in conjunction with neuraminidase, β-galactosidase and β-N-acetylglycosaminidase. In contrast, when this latter digestion is conducted after fucosidase treatment, a fragment of approximate Mr 800 is generated. These findings suggest that in the absence of fucosidase, the following enzymes: β-galactosidase and β-N-acetylglucosaminidase, as well as Endo-D could not act on fraction I. They also suggest that fucosyl residues are blocking the removal of galactose, and N-acetylglucosamine as well as the action of Endo-D.

Fraction II of approximate Mr 1,500 was the main component of cellular glycoconjugate preparations and one of the two major components of cell surface glycopeptides. Inasmuch as fraction II was susceptible to Endo-H showing a reduction in MW of 300 daltons as well as to α-mannosidase treatment, with generation of [2-^{3}H]mannose and it was resistant to neuraminidase, fucosidase, β-galactosidase and β-N-acetylglucosaminidase acting in the presence of Endo-D, this fraction was concluded to be of the "polymannose" type, similar to ovalbumin glycopeptide(s). The loss of 300 daltons is consistent with the removal of the N-acetylglycosaminylasparagine fragment from the glycopeptide, as expected from treatment with Endo-H. Consequently a structure containing $(Man)_6(GlcNAc)_2Asp$ can be suggested for fraction II with the substituted α-mannosyl-residue bound to the C-3 position of the β-mannosyl-residue of the core as suggested by its resistance to Endo-D.

Retinoic acid caused an overall increase of about 60% in the amount of [^{3}H]mannose incorporated into these glycopeptides, without any appreciable effect on the size of the oligosaccharide chains in total cellular glycopeptides.

However, a shift from the lower molecular weight, "polymannose" type glycopeptides to the higher molecular weight "complex" type glycopeptides was found in the cell surface derived material as a result of retinoid treatment. Therefore the change in morphology and adhesive properties towards the "normal" phenotype after retinoic acid treatment may be the result of an overall increase in mannose incorporation as well as a relative increase in the higher molecular weight "complex" type glycoconjugates at the cell surface.

Effect of retinoic acid on EGF-receptors. Retinoic acid treatment of several cell lines enhanced the number of available binding sites for mouse EGF by 6 to 10 fold over controls. This effect was simultaneous with

the effect of RA on adhesion and the biosynthesis of glycosaminoglycans. Only retinoids with biological activity in other systems were active in this system of 3T12 cells.

Significance to Biomedical Research and the Program of the Institute.

Alteration in cell surface adhesion and, in general, in recognition phenomena appears to be a result of neoplastic transformation. Therefore, agents which modify cell surface characteristics to resemble the normal phenotype are of obvious interest in attempts to characterize cell surface traits responsible for invasion, growth in soft agar and other peculiarities of transformed cells.

It has been found that, in addition to their function as chemopreventive agents in chemical carcinogenesis, retinoids can alter cell surface morphology and adhesion of cultured spontaneously-transformed mouse fibroblasts, Balb/c 3T12-3 cells, to resemble the "normal" phenotype. At the molecular level it appears that retinoids modify adhesive properties of transformed cells by their action on the biosynthesis of specific cell surface glycoproteins. Retinoids also seem to affect profoundly the availability of receptors for growth factors, such as EGF-receptors on the cell surface.

Thus, it is reasonable to expect that this project will contribute to our understanding of the basis for the decreased adhesion and greater invasive potential of neoplastically transformed cells and that it will allow a better understanding of mechanisms of growth control.

Proposed course of project.

The following seem to be logical developments of this research: characterization of cell surface glycoproteins responsible for altered growth and adhesion of neoplastically transformed cells; modulation of the synthesis of these glycoproteins by retinoids and antagonism by tumor promoters such as phorbol esters. Study of the mechanism responsible for the increase in EGF receptor sites caused by retinoids.

Publications:

1. Adamo, S., De Luca, L.M., Akalovsky, I., and Bhat, P.V.: Retinoid-induced adhesion in cultured transformed mouse fibroblasts. J. Natl. Cancer Inst. 62:1473-1477, 1979.

2. Sasak, W., De Luca, L.M., Dion, L.D., and Silverman-Jones, C.S.: Effect of retinoic acid on cell surface glycopeptides of cultured spontaneously-transformed mouse fibroblasts (Balb/c 3T12-3 cells). Cancer Res. 1980, in press.

3. De Luca, L.M., Bhat, P.V., Sasak, W., and Adamo, S.: Biosynthesis of phosphoryl and glycosylphosphoryl derivatives of vitamin A in biological membranes. Fed. Proc. 38:2535-2539, 1979.

4. Jetten, A.M.: Retinoids enhance specifically the number of epidermal growth factor receptors. Nature 284: 626-629, 1980.

5. Jetten, A.M.: Action of retinoids on growth and the binding of epidermal growth factor. Ann. N.Y. Acad. Sci. 1980, in press.

6. Jetten, A.M., Meeks, R.G., and De Luca, L.M.: Specific and nonspecific alterations in membrane microviscosity induced by retinoids in embryonal carcinoma and fibroblast cells. Ann. N.Y. Acad. Sci. 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04488-04 LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Carcinogenesis Studies in Cultured Colon from Experimental Animals and Humans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Autrup	Senior Staff Fellow	LEP,NCI
Other:	C. Harris	Assoc. Chief, LEP and Chief, HTSS	LEP,NCI
	A. Haugen	Guest Worker	LEP,NCI

COOPERATING UNITS (if any)
University of Maryland, School of Medicine, Baltimore, MD
National Naval Medical Center, Bethesda, MD

LAB/BRANCH
Laboratory of Experimental Pathology (LEP)

SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A model system for studying chemical carcinogenesis in colon has been developed. Human colon can generally be maintained 14 days and rat colon at least 63 days as explant cultures. Cultured human and rat colon have the ability to enzymatically convert various classes of chemical carcinogens, such as polycyclic aromatic hydrocarbons, N-nitrosamines, mycotoxins and hydrazines, into metabolites which react with cellular macromolecules, such as DNA and protein. A wide variation among people in the binding of both benzo(a)pyrene (BP) and 1,2-dimethylhydrazine (DMH) was seen. The data showed a skewed distribution which strongly suggested a distribution more complex than uni-modality. The carcinogen-DNA adducts for aflatoxin B_1 (AFB), BP, N-nitrosodimethylamine and DMH have been identified and the persistence of the AFB-DNA modification have been studied in human colon. The effects of various suspected inhibitors and enhancers of colon carcinogenesis have been investigated on the metabolism of BP and DMH.

PHS-6040
(Rev. 10-76)

Objectives: To develop a model system for studying carcinogenesis in human colon. To compare the metabolism and the effect of chemical carcinogens in human colon to that in experimental animals. To study the effect of various co- and anti-carcinogens on the metabolism of these carcinogens.

Methods Employed: Explant cultures of colon; autoradiography, xenotransplantation; electron microscopy; high resolution light microscopy; isolation of cellular macromolecules; high pressure liquid chromatography; thin layer chromatography.

Major Findings: Human colonic epithelium has been cultured as explants in a chemically defined medium for periods up to 24 days. The viability of the explants was shown by the preservation of the ultrastructural features of the colonic epithelial cells and by active incorporation of radioactive precursors into cellular DNA and protein. The ability to maintain colonic mucosa in culture was subject to both intra- and inter-individual variation. The explants were generally maintained up to 14 days under these conditions. Adult rat colon was maintained with a modified structural integrity for up to 63 days under similar conditions.

Metabolism of various carcinogens in cultured human colon has been investigated. Non-tumorous colonic tissue was collected at the time of either "immediate autopsy" or surgery from patients with or without colonic cancer (170 cases). After 24 hrs in culture, explants were exposed to radioactive-labeled carcinogen for another 24 hrs and the binding to cellular DNA was measured by radiometric methods.

The binding levels of BP to DNA showed a skewed distribution which strongly suggests more than uni-modality. The mean binding level is 6.3 pmoles/10 mg DNA (median 4.3; range 0.2-39.3). There was no significant difference in binding level between surgical tissue obtained from colon cancer patients (mean 7.1; median 3.5), surgical tissues from non-colon cancer patients (6.4; 4.1) or from immediate autopsies (7.3; 5.4). The major adduct was formed by trans-addition of BP diol epoxide I to the exocyclic 2-amino group of guanine. The binding of BP to DNA was inhibited by addition of antioxidants, butylated hydroxytoluene and disulfiram to the media, while coincubation with either lithocholic acid (1 μM) or chenodeoxycholic acid (2.5 μM) significantly increased the binding value of BP. These bile acids caused an enhancement of the overall BP metabolism. None of the other bile acids--cholic acid, deoxycholic acid, taurodeoxycholic acid--showed any persistent effect on BP binding. A positive correlation between the binding of BP and DMH to DNA was found ($r = 0.500$; $p < 0.001$; 127 cases). The binding value of DMH to DNA also showed a wide variation among people, the distribution being similar to that of BP binding to DNA. Slightly higher mean binding levels was seen in tissue from immediate autopsies than from surgical biopsies. No significant age difference was observed, while females (age group 51-70; sigmoid colon) have a higher binding level than men. When the anatomical segments were compared, the highest binding of DMH was observed in the sigmoid and transverse colon and the lowest in rectum and the descending colon. A higher binding level was found in duodenum than in transverse colon from the same patient.

AFB, a colonic and hepatic carcinogen in rats, is metabolized by cultured human colon. The mean binding level of AFB to DNA (1.8 + 2.0) is lower than that of BP (5.9 + 5.7) in the same patient. No correlation was found between the two sets of binding levels ($r = 0.136$; $p > 0.05$; 24 cases). One major AFB-DNA adduct was formed by addition of AFB-2,3-oxide to the 7-position of guanine. This reaction product is unstable and the imidiazole ring will open to stabilize the molecule. This ring opened form is the major adduct when human colon was cultured for up to 3 days after AFB treatment for 1 day.

The effect of treatment of the colon explant with a direct acting carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) has been investigated. A concentration of 0.3 μg/ml induced unscheduled DNA-synthesis and no toxic morphological response was seen 7 days after a single treatment with MNNG (3 μg/ml). Xenotransplantation of explants treated 3 times with 0.3 μg/ml developed squamous metaplasia, whereas the controls which were treated with the vehicle only did not show any morphological changes.

Significance to Biomedical Research and the Program of the Institute: A colon explant system has been developed which can be used for carcinogenesis studies. Chemical carcinogens which are metabolically activated and form carcinogen-DNA adducts by human colon may induce cancer in this tissue in humans. Determination of binding levels of carcinogens in a greater group could identify people at high risk for chemical induction of colonic cancer. The model system provides a link between experimental animal studies and the human situation, as comparative studies of animal and human tissues can be performed under similar controlled conditions. Human tissues obtained by immediate autopsy also allow a comparative study in various organs from the same individual.

Proposed Course of the Project: To continue improving the culture conditions for human colon. The effect of exposure of the explants to carcinogens and potential promotors of colon carcinogenesis will be investigated both biochemically and morphologically, using combinations of explant cultures and xenotransplantation. The metabolism of AFB and N-nitrosopyrrolidine will be investigated in more detail. The relationship between the genetic control of DMH-carcinogenesis and the enzymatic activation of DMH will be investigated in an animal model.

Publications

Autrup, H., Schwartz, R.D., Essigmann, J.M., Smith, L., Trump, B.F., Harris, C.C.: Metabolism of aflatoxin B_1, benzo[a]pyrene and 1,2-dimethylhydrazine by cultured rat and human colon. Teratogenesis, Mutagenesis and Carcinogenesis, in press, 1980.

Autrup, H.: Explant Culture of Human Colon. In Harris, C.C., Trump, B.F., and Stoner, E.D. (Eds.): Cultured Human Tissues and Cells in the series Methods in Cell Biology. New York, Academic Press, 1980, Vol. 21B, pp. 385-401.

Autrup, H., Jeffrey, A.M., and Harris, C.C.: Metabolism of Benzo(a)pyrene by Cultured Human Tissues. In Bjorseth, A., and Dennis, A.J. (Eds.): Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects. Columbus, Battelle Press, 1980.

Autrup, H., Harris, C.C., Schwartz, R.D., Trump, B.F., Smith, L.: Metabolism of 1,2-dimethylhydrazine by cultured human colon. Carcinogenesis, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04490-04 LEP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Evaluation and Prevention of Carcinogenic Effects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Umberto Saffiotti, M.D., Chief, Laboratory of Experimental Pathology, DCCP, NCI

COOPERATING UNITS (if any)

The U.S. Regulatory Council, the Interagency Regulatory Liaison Group (IRLG), the Occupational Safety and Health Administration (OSHA) and other federal agencies.

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary objectives of this work are to examine current knowledge in the field of carcinogenesis and related fields and to identify criteria for the evaluation of carcinogenic effects of chemical and physical agents, for the evaluation of risk assessment methods, and for the prevention of cancer hazards in the human population.

Laboratory methods and biological models for the detection of carcinogenic activity of chemicals are examined and evaluated, with particular emphasis on animal models of carcinogenesis and in vitro models for carcinogenesis studies.

Criteria for the evaluation of carcinogenic risks from environmental and occupational human exposures are developed. Research approaches are outlined for studies in carcinogenesis mechanism addressed to the problems of occupational and environmental cancer prevention.

PHS-6040
(Rev. 10-76)

Objectives: To examine current knowledge in the field of carcinogenesis and related fields, to identify criteria for the evaluation of carcinogenic hazards in the human population and for assessment of their risk, and to develop research approaches relevant to the study of occupational and environmental cancer prevention.

Methods Employed: Examination and evaluation of biological models, methods and findings used for the detection of carcinogenic activity and the identification of mechanisms of carcinogenesis. Analysis of results of carcinogenesis studies, including experimental design, metabolism, pathology and statistical evaluations. Analysis of occupational and environmental exposure data and of interactions of carcinogens with human tissues. Analysis of regulatory, legislative and societal approaches. Development of critical reviews and documentation in environmental carcinogenesis. Organization of and participation in conferences and workshops to provide evaluation of documentation on these topics. Participation in advisory groups for agencies of the Federal Government and for national and international organizations.

Major Findings: Particular contributions during this year were provided in the following areas:

a) For the U.S. Regulatory Council: Review of criteria and scientific bases used for the regulation of carcinogens by federal agencies and participation in the development of a unified policy, approved by the relevant federal agencies and by the President and issued in October 1979. This policy is based on the review "Scientific bases for the identification of potential carcinogens and estimation of risks" prepared by the IRLG Working Group on Risk Assessment (1979), and on other governmental reports and activities. The Regulatory Council's working group was instrumental in developing a national policy consistent with current scientific knowledge and with public health and economic goals.

b) For the Interagency Regulatory Liaison Group (IRLG): Participation has continued in the Working Group on Risk Assessment, particularly to review the comments received on the extensive report published in July 1979.

c) For the Occupational Safety and Health Administration (OSHA), U.S. Department of Labor: Extensive review of documentation and advice on criteria for the evaluation and classification of occupational carcinogens were provided to OSHA during the preparation of the general policy for the identification and regulation of physical and chemical substances that pose a potential occupational carcinogenic risk to humans. The final OSHA policy was approved after over three years of extensive public debate, review, hearings, and analysis of scientific literature and other material for a total Record consisting of over 250,000 pages. The policy "Identification, Classification and Regulation of Potential Occupational Carcinogens" was issued by OSHA on January 22, 1980. Since that time, advice was provided to OSHA on the implementation of the policy and the criteria for the selection of the

"Candidate List" of compounds to be considered for in-depth review in order to arrive at a "Priority List" of substances for which OSHA will develop regulations. This advisory role is now conducted through an NCI working group chaired by U. Saffiotti.

Additional collaboration with OSHA was provided through participation in the OSHA Seminar "Lost in the Workplace: Is There an Occupational Disease Epidemic?" (Chicago, September 1979) and in the "Swedish-American Conference on Chemical Hazards" (Washington, D.C., March 1980).

d) For the U.S. Environmental Protection Agency (EPA): 1) Review and advice on the EPA Genetic Toxicology Program through membership in its Assessment Panel. 2) Review and advice on the program on short-term in vivo carcinogenesis tests. 3) Advice on the development of the NCI-EPA collaborative research program.

e) For the Consumer Product Safety Commission: Review and evaluation on the carcinogenic and toxic effects of formaldehyde.

f) For the National Institute of Occupational Safety and Health (NIOSH): Collaboration in the development and review of the NCI-NIOSH collaborative research program, and as Co-Project Officer on several research projects.

g) Review of state of knowledge and recommendations of criteria for the evaluation of carcinogenic effects: (1) Pesticides (review in press, 1980); (2) Metals (working paper and report of conference, March 1980); (3) Carcinogenesis studies related to occupational cancer (three papers at conference in Helsinki, Finland, August 1979); (4) Carcinogenesis studies related to to epidemiologic methods (paper at SOEH conference, December 1979).

h) International activities: (a) Invited to visit carcinogenesis laboratories in the USSR (Leningrad, Kiev and Moscow), to lecture and discuss mutual research interests (August 15-27, 1979) under the auspices of the US-USSR Cancer and Environmental Health Programs; (b) Invited lecturer, course on Occupational Cancer, Helsinki, Finland, August 1979; (c) Participant in joint meetings, U.S.-Italy Cancer Program, visits to Italian laboratories and to the Istituto Superiore di Sanita, Rome; representative of the Director, NIH, and invited speaker in the Conference on Italian Biomedical Scientists in the United States, Rome, National Research Council, April 1980.

i) Other professional activities include (1) service as President (1978-1982) of the Society for Occupational and Environmental Health; (2) Participation in the organization of a Symposium on Teratogenesis, Carcinogenesis and Mutagenesis, Hunt Valley, MD, September 1979; (3) Editorial activity as Associate Editor of "Teratogenesis, Carcinogenesis, Mutagenesis" and member of the editorial board of the Journal of Environmental Pathology and Toxicology; (4) Member of the working group on mutagenic burden, NCI; (5) Invited lecturer in several institutions.

j) Recognition received this year includes the Public Health Service Special Recognition Award, 1980; and election to Fellow of the New York Academy of Sciences.

Significance to Biomedical Research and the Program of the Institute: The national policies on environmental health and cancer prevention need to be based on sound scientific grounds. A solid and well documented basis of research data and evaluations is necessary in the development of criteria for sound health protection policy. The experience obtained by analyzing and coordinating different methodological approaches to the study of the carcinogenic process - at the human, animal, cellular and molecular level - provides a strong basis for identifying specific criteria and priorities for public health and for further research.

This year, with the issuance of the IRLG and Regulatory Council Reports and of the OSHA carcinogen policy, the development of a sound federal policy for the control of environmental carcinogens reached a milestone which reflects current coherent scientific criteria. The evolution of scientific knowledge will necessitate a process of continuous evolution in these criteria.

Proposed Course of Project: Continuation of these activities.

Publications

Saffiotti, U.: Review of pesticides carcinogenesis data and regulatory approaches. In Davis, W. and Rosenfeld, C. (Eds.): Carcinogenic Risks. Strategies for Intervention. Scient. Publ. No. 25, International Agency for Research on Cancer, Lyon, 1979, pp. 151-166.

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Saffiotti, U.: From Panel Discussion I. Scientific uncertainties in evaluation of environmental disease. In Hammond, E.C. and Selikoff, I.J. (Eds.): Public Control of Environmental Health Hazards. Ann. N.Y. Acad. Sci. 329: 165-167, 1979.

Saffiotti, U.: (On the nature and identification of carcinogens). From Panel "Of mice and men: cancer in the workplace." In: Lost in the Workplace: Is There an Occupational Disease Epidemic? U. S. Department of Labor, Occupational Safety and Health Administration, Washington, D.C., 1979, pp. 309-317.

Saffiotti, U. and Cortesi, E.: Carcinogenicity of pesticides: Review and evaluation of recent results. In Kimbrough, R. and Kahn, E. (Eds.): Pesticides and Human Health. Society for Occupational and Environmental Health, Washington, D.C., 1980. (In press).

Reports

Interagency Regulatory Liaison Group, Working Group on Risk Assessment: Scientific bases for identification of potential carcinogens and estimation of risks. J. Natl. Cancer Inst. 63: 241-268, 1979. (and Fed. Register 44 (No. 131): 39858-39879, July 6, 1979).

Regulatory Council: Statement on regulation of chemical carcinogens; Policy and request for public comments. Fed. Register 44 (No. 202): 60038-60049, October 17, 1979.

Department of Labor, Occupational Safety and Health Administration. Identification, classification and regulation of potential occupational carcinogens. Fed. Register 45 (No. 15): 5001-5296, January 22, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04491-04 LEP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Quantitative studies on concurrent exposures to numerous carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Umberto Saffiotti	Chief	LEP, NCI
Other:	Jerry M. Rice	Chief, Perinatal Carcinogenesis Section	LEP, NCI
	Winston D. Edwards	Visiting Associate	LEP, NCI
	Enrico Cortesi	Visiting Fellow	LEP, NCI
	Paul J. Donovan	Chemist	LEP, NCI
	Takeo Kakunaga	Chief, Cell Genetics Section	LMC, NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Experimental Pathology

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NCI, NIH, Bethesda, Maryland 20205

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2.5	1.9	0.6

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Experimental studies are conducted on concurrent exposure of appropriate biological targets to numerous carcinogens of different classes, each administered at doses which would be expected to produce low or undetectable effects in the chosen system. Quantitative exposure/response studies are addressed to test the hypothesis that many different carcinogens can act synergistically to induce a significant level of carcinogenic response when given concurrently at individually subeffective doses. Mechanisms of action are studied to determine the most effective conditions of synergism. The biological models selected for these studies include in vitro assays for mutagenesis and neoplastic cell transformation and whole animal models. Analysis of dose-response relationships in carcinogenesis is conducted on data collected in a comprehensive literature survey, and on dose-response results obtained experimentally in the selected biological systems.

PHS-6040
(Rev. 10-76)

Objectives: To determine whether a marked carcinogenic effect can result from concurrent exposure to numerous different carcinogens, each administered at doses which would be expected to produce low or undetectable effects in the chosen target system; and to analyze interactions of effects and dose-response relationships in carcinogenesis.

Methods Employed: Biological model systems include the Salmonella mutation assay (Ames) and neoplastic transformation of Balb/c 3T3 mouse fibroblasts in culture (Kakunaga). Whole animal carcinogenesis sudies are planned. Quantitative exposure protocols were used for graded doses of individual carcinogens and their combinationns (up to 8 compounds together so far). The results were analyzed quantitatively.

I. Salmonella mutagenesis: Strains TA 98 and TA 100, responding respectively to frame-shift and to base substitution mutagens, were used with or without S-9 mix prepared from Aroclor 1254-induced rat liver.

II. Neoplastic transformation: The Balb/c 3T3 clone A31 mouse fibroblast cell line system was used. The clones were obtained from T. Kakunaga, Laboratory of Molecular Carcinogenesis, DCCP, NCI. Most studies were performed on clone A31-1, and a few on clone A31-13.

The cell system was set up following the original protocol (Kakunaga, Intl. J. Cancer 12: 463-473, 1973) with minor modifications. Culture conditions and use of media (MEM with 10% fetal bovine serum) and solvents were optimized. Each study included toxicity determinations (8 days) and neoplastic transformation experiments (5 weeks). Scoring of transformation results was based on the number of Type III foci, but the induction of Type II foci was also recorded. Twenty-four hours after plating 10^4 cells per 60mm Petri dish, chemicals under study were added to the medium and removed 72 hours later. No metabolic activators were added. Medium was subsequently changed twice per week.

Major Findings: I. Salmonella mutagenesis. In the previous year, mutual inhibition was demonstrated for a mixture of 5 polycyclic aromatic hydrocarbons as well as for each individual combination of components; mutual synergism was demonstrated for a group of compounds belonging to different chemical classes: benzo[a]pyrene (BP), aflatoxin B_1 (AFB), benzidine (BZ) and safrole (SF), while ethylene thiourea was inactive. Continuation of these studies confirmed the synergistic effects of this group of compounds and their individual combinations. Synergism occurred at low levels of exposure where toxicity is minimal for all compounds, and over a range of concentrations of the metabolically active S9 mix. The questions was considered whether compounds of the same chemical class would be likely to produce mutual inhibition because of competitive mechanisms while compounds of different classes would be more likely to act synergistically. A different class of compounds was therefore chosen for further study, the aromatic amines. This class was selected because of: a) data on carcinogenic effects in humans

and in animals, b) the synergistic effects of benzidine previously observed, and c) the recent findings of high carcinogenic activity for benzidine dyes and their metabolic breakdown to several free aromatic amines.

The following aromatic amines were studied in TA 98 and TA 100: Benzidine (BZ), 3,3'-dichlorobenzidine (DCB), 4-aminobiphenyl (4-AB), 2-naphthylamine (2-NA), 1-naphthylamine (1-NA), o-dianisidine (OD), o-tolidine (OT), and aniline (AN). The first four of these compounds are well known carcinogens, while the latter four compounds have low or negative activity. Dose response mutagenicity data were obtained for all compounds except o-tolidine and aniline, which were found not mutagenic. BZ, 4-AB, 2-NA and 1-NA required metabolic activation by S9, but DCB and OD were mutagenic even without S9, although S9 greatly enhanced their effect. DCB was more active in TA 98, while 4-AB and 2-NA were more active in TA 100. Combination tests were conducted at equimolar doses (0.125 M/plate) with S9. Various permutations of combinations were studied, including mixture of the 8 compounds (resulting in inhibition in both strains), mixture of the 3 most active compounds, DCB, 4-AB and 2-NA (inhibition in TA 100 and additive effect in TA 98) and permutations of 2 compounds at a time: most of these showed approximately additive effects, a few were inhibitory (4-AB + OD and 4-AB - OT, in both strains), while the combination BZ+4-AB resulted in synergism in both strains. Tests of the benzidine dyes were conducted with S9 and riboflavin additions: direct black 38 was found highly mutagenic in TA 98 by the plate assay (15 times higher than background) and 10 times more active than that when tested by liquid incubation. Direct brown 95 and direct blue 6 were not active in any of these tests. It was noted with interest that direct black 38 was found to yield both BZ and 4-AB by metabolic breakdown; synergism of metabolically released free bases is suggested as a possible factor in the high carcinogenic effect of the dye. On a molar basis, direct black 38 was found to be about 5 times more active in plate assays than the amount of benzidine contained in the dye molecule.

Studies of combinations of aromatic amines with other carcinogens are under way.

II. Neoplastic Transformation in Balb/c 3T3 clone A31 cells. These studies, initiated in the Spring of 1979, have given so far the following results. Dose-response relationships for transformation were determined for the following reference compounds: N-methyl-N'-nitro-N-nitrosoguanidine, 4-nitroquinoline-N-oxide, 2-fluorenylacetamide, 3-methylcholanthrene 7,12-dimethylbenz[a]anthraceneand BP. A group of compounds, previously found to act synergistically in the Salmonella mutagenesis system, was studied for cytotoxicity and transformation; dose response relationships for transformation were determined for BP, BZ and AFB while safrole and ethylenethiourea did not induce transformation in these tests. Another group of compounds, the aromatic amines, is under study: the same compounds listed above for mutagenesis were also selected for cytotoxicity and transformation studies, which are still under way.

Combined effects in this system of cells in culture were studied for BP, BZ and AFB. Results of toxicity tests generally indicate an additive pattern of cytotoxic response for the combined exposures so far studied; transformation experiments are underway.

III. Data review and analysis. Data on dose-response relationships in carcinogenesis were collected for a comprehensive survey. Their analysis was continued based on class of carcinogens, route, dose and number of administrations. Particular emphasis was given to studies of multiple exposures to different agents.

Significance to Biomedical Research and the Program of the Institute: Multiple concurrent exposures to many different carcinogens represent the actual conditions under which the human population is exposed to carcinogens. From prenatal life through childhood and adult life people are exposed to a large number, probably in the hundreds, of environmental carcinogens from different routes. Such "realistic" conditions of exposure have never been reproduced experimentally so far. The definition of the effect of this background low-level exposure to individual carcinogens, acting together with the exposure to many others, is expected to provide information on the mechanisms of carcinogenesis in tissues exposed to multiple hits by different chemicals and on the role of multiple synergisms in carcinogenesis. The role of high-level exposures to single carcinogens, when superimposed on different kinds of multiple background exposures will also be studied. Wide-ranging implications can be projected for the understanding of basic chemical biological interactions in carcinogenesis, for mathematical models for dose/response extrapolation and for the evaluation of carcinogenic hazards in public health policies.

Proposed Course of Project: Continuation and extension of these studies in biological systems for mutagenesis, cell transformation and in vivo carcinogenesis, using additional types of carcinogens, until a large group of synergists is defined; mechanism studies will be further developed to elucidate the bases of interactions and their relation to the human response.

Publications

Saffiotti, U., Rice, J.M. and Donovan, P.J.: Interactions of multiple carcinogens at low levels of exposure: preliminary mutagenicity studies using the Ames Salmonella system. In Santi, L. and Parodi, S.(Eds.): Short-Term Tests for Prescreening of Potential Carcinogens. Istituto Scientifico per lo Studio e la Cura dei Tumori, Genoa, 1979, pp. 99-109.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04504-08-LEP

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Model Systems for the Study of Chemical Carcinogenesis at the Cellular Level

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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9	5	4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse epidermal cell cultures and epidermal cell lines are utilized as models for studying mechanisms of epithelial carcinogenesis in vitro. Low extracellular Ca^{++} concentration in culture medium selects for proliferating cells while elevated Ca^{++} induces terminal differentiation. Carcinogen exposure results in the development of cell colonies which are resistant to Ca^{++} induced terminal differentiation. Tumor promoters, ultraviolet light and chemical agents which produce DNA breaks induce ornithine decarboxylase in epidermal cells. Retinoids inhibit tumor promoter induced ornithine decarboxylase prior to activation of enzyme transcription while corticosteroids, local anesthetics and protease inhibitors, act at a site subsequent to gene activation. Tumor promoters also induce epidermal transglutaminase. Antibodies to adducts resulting from covalent binding of the carcinogens acetylaminofluorene and benzo[a]-pyrene to DNA have provided sensitive (< 1 picomole) and specific immunoassays to quantitate these binding products in cells and tissues. Such assays can detect quantitative or qualitative alterations in binding in different cell types or under differing exposure conditions.

Objectives: To study cellular and molecular changes during stages of chemical carcinogenesis through the use of unique in vitro model systems designed to simulate well studied in vivo models. Studies are directed to give insight into general changes occurring in mammalian cells during malignant transformation and specific molecular events which may be causative to the transformation process. Specific markers of the transformed phenotype are also being sought and mechanisms to prevent or reverse transformation are being studied.

Methods Employed: This laboratory has developed and utilized mouse epidermal cell culture as a major model to approach the stated objectives. Previous studies have shown that this model functions biologically in a highly analogous fashion to mouse skin in vivo. Human epidermal cells obtained from neonatal foreskins have also been adapted to growth in vitro in recent years. In vivo studies utilizing the two-stage mouse skin carcinogenesis model and grafts of human or mouse skin into nude mice are also employed. A number of laboratory techniques are required to pursue the objectives. Morphology is followed by light and electron microscopy and histochemical staining. Macromolecular synthesis and growth kinetics are studied by biochemical and autoradiographic procedures. Cellular functions, including the production of specific differentiation products, are monitored by enzyme assays, gel electrophoresis, amino acid analysis and radioimmunoassay. The progression to the malignant phenotype is monitored by growth rates, soft agar assay, karyotypic abnormalities, enzymatic changes and injection of cells into nude or newborn mice. A number of immunologic techniques including cell surface antibody production, fluorescent staining, and immunoprecipitation are being performed to recognize the normal or altered phenotype. Radioimmunoassay is used in studies of differentiation and carcinogen-DNA binding and repair. In addition the chemical synthesis of DNA-carcinogen adducts and their purification by column chromatography are currently performed. Isolation of macromolecules for carcinogen binding studies and assays for repair and DNA molecular weight determinations utilize density and sedimentation velocity gradient centrifugation.

Major Findings: The pursuit of this project has led to major new findings in three pertinent areas: 1) enhancement of model systems including improved culture methodology, transformation assay, and the identification and selection of preneoplastic cells; 2) unique assay techniques for carcinogen-DNA interactions and new information concerning the biochemical and biological consequences resulting from carcinogen exposure; 3) increased understanding of the process of preneoplastic progression and the mechanism of tumor promotion and anti-promotion. In addition confirmation of in vitro results by in vivo experimentation has been obtained.

During the past year, both our understanding of the fundamental biology of the mouse epidermal culture system and the usefulness of this model for carcinogenesis studies have been greatly enhanced. The recent discovery that ionic calcium is a critical regulator of epidermal growth and differentiation have provided a method to control and study each of these phases

of the keratinocyte life cycle. At low ionic calcium concentrations in culture medium (0.02 - 0.09 mM), epidermal cells maintain a monolayer growth pattern with a high proliferation rate. Essentially 100% of the attached cells are in the proliferating cell pool. Differentiation continues but differentiated cells are discarded into the culture medium. Proliferating keratinocytes synthesize prekeratins and display the basal cell pattern of differentiation proteins by polyacrylamide gel electrophoresis.

Ultrastructure studies indicate that tonofilaments are abundant but are arranged in a perinuclear fashion. Cell surfaces are adjoined by tight and gap junctions but desmosomes are absent. The cell surface of many cells has a microvillous or ruffled pattern. Mitotic cells are in great abundance. Immunofluorescent studies have indicated that cells in low calcium contain abundant bullous pemphigoid antigen, a molecule associated with the basal layer in skin sections from all mammalian species. Absent from these cells is pemphigus antigen and stratum corneum basic protein, two molecules associated with the differentiated layers of epidermis. Cells grown in low calcium medium have very low activity of the enzyme epidermal transglutaminase, the enzyme responsible for the formation of the cornified envelope crosslink in differentiated keratinocytes. Likewise the % of cross-links (measured as lysine found as the dipeptide ε-amino[γ-glutamyl]lysine relative to total lysine) measurable in epidermal cell monolayers cultured in low calcium medium is less than 10% of that found in differentiated epidermis.

When cells maintained under low calcium growth conditions are switched to medium with calcium contents above 0.1 mM (standard culture media are 1.2 - 1.8 mM), differentiation is induced. The earliest changes are observed by electron microscopy. Within 5 minutes new hemidesmosome formation can be observed and by 1 hour complete desmosomal connections with associated tonofilaments are abundant. Within 24 hours dense cell colonies begin to stratify, form keratohyalin granules and cornified envelopes and within 48-72 hours sheets of mature squamous cells detach from the culture dish. When macromolecular synthesis is studied, one finds that DNA synthesis is completely inhibited by 36 hours after addition of calcium. In contrast, RNA and protein synthesis only gradually decrease over a 72 hour period. By the use of a double labeling technique with the amino acids histidine or leucine (^{14}C and ^{3}H), specific changes in protein synthesis associated with differentiation have been determined by polyacrylamide gel electrophoresis. During the first 48 hours after switching from .07 to 1.2 mM Ca^{++}, specific increases in the synthesis of proteins in the 30K - 50K region can be detected. One of these proteins appears to be actin but the others are as yet unidentified. There is no alteration in the synthesis of keratin proteins. Within 6 hours of the elevation of calcium, epidermal transglutaminase activity rises and reaches levels 2-3 fold above basal activity by 72 hours. During this same time period there is a 2-4 fold increase in the percentage of crosslinks of the ε-amino(γ-glutamyl)-lysine type. By immunofluorescence detection, bullous pemphigoid antigen disappears and pemphigus antigen appears during calcium induced differentiation. Likewise granules appear which show strong fluorescence with stratum

corneum basic protein antibody. All of the foregoing data imply that low calcium culture conditions select for the basal cell population while higher calcium levels induce differentiation in a manner closely resembling the in vivo process.

The mechanism by which calcium regulates epidermal differentiation has been studied in some detail. The induction appears to be specific for Ca^{++} ions since Ni^{++}, Cd^{++}, Co^{++}, Cu^{++}, Mn^{++}, Zn^{++}, Li^{+}, Sr^{++}, or La^{+++} failed to reproduce or inhibit the Ca^{++} induced effect. Organic cationic compounds such as methylamine, putrescine and ammonium chloride also were without effect. Inhibition of protein synthesis by cycloheximide, RNA synthesis by actinomycin D or DNA synthesis by hydroxyurea at the time of the calcium increase, failed to significantly alter the induction of differentiation. Microtubule and microfilament disrupting agents such as colcemid and cytochalasin B also did not interfere with calcium induced differentiation. Drugs which are known to antagonize intracellular calcium mediated reactions such as local anesthetics and quercitin did not affect calcium induced differentiation. A variety of agents which are agonists, activators or inhibitors of cyclic nucleotides did not induce or inhibit the induction of differentiation. Furthermore, cAMP and cGMP levels were determined by radioimmunoassay both before and shortly after addition of Ca^{++} and did not change. These studies did not indicate a mechanism but suggested that the effects of calcium were quite specific and likely direct rather than through activation or induction of a mediator. Further studies have suggested that the cell membrane may be the site of the calcium effect. Two agents have been studied which can either partially or completely block calcium induced differentiation. The calcium ionophore A23187 and the inhibitor of the Na-K pump, ouabain, are effective inhibitors of all of the morphological changes associated with Ca^{++} addition. These effects occur at non-toxic doses and suggest that membrane charge potential, membrane ion flux or other effects at the cell surface, rather than elevation of intracellular calcium, are responsible for the induction of differentiation.

The capacity to induce terminal differentiation provides a model to study the role of physiological modulators of that process. Retinoids have been shown to alter epidermal differentiation in vivo and in vitro. Retinoids prevent or delay terminal differentiation induced by calcium as judged by vertical stratification, granule formation and the increase in transglutaminase activity. Paradoxically, transglutaminase activity is elevated by retinoids in low calcium conditions but the rise in activity after calcium elevation does not occur. The amount of ε-(γ-glutamyl)lysine crosslinks formed after calcium elevation is also inhibited by retinoids. Ultimately, retinoid treated cells switched to high calcium are sloughed into the culture medium but never as mature squames. Thus retinoids can alter the process of terminal differentiation but cannot prevent the ultimate death of the epidermal cell.

The regulation of epidermal proliferation and differentiation by calcium forms the basis of a new in vitro assay developed for detecting carcinogen induced changes. Reasoning that during the process of carcinogenesis an alteration in differentiation must occur, we treated epidermal cells grown in low calcium medium with carcinogens and counted colonies which were resistant to calcium-induced cell death by terminal differentiation at a later time. Colonies selected in high calcium were fixed and stained with rhodamine, a stain for keratinizing colonies. To date, results of studies with two carcinogens N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz[a]-anthracene (DMBA) are available. In 5 experiments, representing a total of 319 cultures, the number of continuously growing colonies selected from carcinogen treated cultures exceeded that in controls (spontaneous variants) by as much as 30 fold with an average of 8 fold. Dose response studies with both MNNG and DMBA reveal an increase in transformation frequency with dose of carcinogen when correction for toxicity is made. Reproducibility within an experiment is excellent for the same dose of carcinogen. Colonies derived in this way are generally discreet and quantifiable. All colonies are composed of epithelial cells with characteristic epidermal markers. Such cells do not grow in agar and have not produced tumors in animals within 6 months after subcutaneous injection into syngeneic newborns. Thus this assay may select for preneoplastic cells which arise spontaneously in epidermal cell cultures or which can be induced by carcinogens. Alternatively, these changes may be unrelated to the process of carcinogenesis. Studies are in progress to determine if cells selected in this manner progress to the fully malignant stage either spontaneously or by further treatment with carcinogens or tumor promoters. To improve this assay further, techniques have been developed to culture epidermal cells under clonal growth conditions. A variety of culture methods have been tested. Optimum conditions employ dermal fibroblast-conditioned, low calcium medium and epidermal growth factor. Cloning efficiencies of 1-6% can be achieved routinely with primary or secondary cultures of keratinocytes. Subclones have been derived with higher cloning efficiencies but which still require conditioning factors and EGF. These subclones are presently being utilized to develop a clonal transformation assay similar to the mass culture assay described above. Alterations induced by carcinogens have also been detected via the clonal assay since such cells have a higher cloning efficiency within 4 weeks of exposure when compared to controls. Further studies are directed at determining how soon this increased cloning efficiency can be detected. Preliminary studies also suggest that carcinogen treated cells are capable of continued proliferation in culture medium of very low calcium content (<0.02 mM) while controls do not survive these conditions. Thus, a series of selection pressures can be utilized to recognize epidermal cell clones altered by carcinogens.

A critical element in the development of transformation assays for epithelial cells is the identification of markers of malignancy. In the previous year this laboratory developed a series of epidermal cell lines which had spontaneously transformed in culture. These lines produced squamous

cell carcinomas upon injection into syngeneic mice. These lines were examined in great detail to identify characteristics associated with malignancy. Only 1 of 12 lines grew in agar, and the use of agarose or methocel failed to improve anchorage independent growth. All lines studied retained differentiative characteristics including the synthesis of keratin proteins, near normal protein patterns by gel electrophoresis and the production of pemphigus, pemphigoid and stratum corneum basic protein antigens. Endogenous activity of the enzyme ornithine decarboxylase, reported to be elevated in epidermal carcinomas in vivo, was variable in these lines as was the induced level after exposure to TPA. The only consistent alteration noted was the behavior of all lines to changes in medium calcium concentrations. Whereas low calcium conditions resulted in identical monolayer behavior as seen in normal epithelial cells, a switch to high calcium failed to induce cell death associated with terminal differentiation. Thus a defect in calcium-induced differentiation, similar to that employed to select carcinogen treated primary cultures (thought to be preneoplastic variants), was reproduced in these malignant lines. When a small number of malignant cells was mixed with a larger population of normal cells and grown under low calcium conditions, selection in high calcium resulted in colonies derived from the malignant cells whereas controls (with no added transformed cells) failed to yield colonies. This provides support for the use of calcium selection as a basis for a transformation assay in epidermal cell culture.

Building on our advances in mouse epidermal cell culture, we have applied new techniques to culture and characterize human epidermal cells. Keratinocytes from neonatal foreskins can now be maintained in culture for 4-5 months and undergo up to 9 subcultures with a potential population doubling of 730 fold. Using a colony assay developed in this laboratory, optimum culture conditions were established as follows: medium 199 with 0.4 mM calcium, 10% fetal calf serum; pH 7.0 - 7.2; temp 36°; rat tail collagen substrate. More recently, lower calcium levels have been utilized in combination with medium supplementation with cholera toxin and epidermal growth factor. It is hoped that a transformation assay utilizing calcium selection can be developed for human epidermis as well. The functional integrity of human keratinocytes for up to 6 subcultures under optimized conditions has been studied by immunofluorescence. Such cultures are positive for keratin, pemphigus, pemphigoid and laminin antigens but negative for type 1 collagen and factor VIII antigen. Initial experiments to determine the reaction of human keratinocytes to phorbol ester tumor promoters have indicated that a new cell type with characteristics unlike either keratinocytes or fibroblasts is stimulated to proliferate. Studies to identify the origin of this cell type are in progress.

The detection and quantitation of epidermal specific markers have been a goal of this Project since it is hoped that changes in tissue specific markers will be found to be associated with malignancy. A number of immunofluorescent and electrophoretic approaches have already been outlined. During the past year, we have developed a quantitative technique for the assay of the major epidermal differentiation protein,

keratin. By iodinating mouse keratin protein, a radioimmunoassay (RIA) has been developed which can detect nanograms (picomoles) of keratin proteins in cell and tissue extracts. Essentially 100% of mouse keratin can be quantitated when anti-mouse keratin antiserum is used in this assay. Human, bovine, rat and hamster keratin are also quantifiable but with a substantially lower efficiency than that of mouse protein. A survey of extracts from a variety of mouse tissues and organs has indicated that keratin proteins are an important component of epithelium from organs other than skin. Malignant epidermal cells also contain keratin proteins. Keratins have been quantitated in cultures of human skin and bronchial epithelium by this assay. It is hoped that the keratin RIA, which is the most sensitive procedure available to assay these proteins, will aid in future studies to detect a functional impairment of the keratin gene which may occur after carcinogen treatment.

Studies to understand the mechanism of tumor initiation have attempted to determine the basis for strain susceptibility to skin carcinogenesis and have also focused on the interactions of carcinogens with DNA. The Sencar mouse strain was bred for susceptibility to two-stage skin carcinogenesis. Previous studies had indicated that Sencar mice were susceptible to several classes of initiators, that newborn Sencar mice were more sensitive to skin carcinogenesis than newborn Balb/c mice, and that tumor promotion alone induced a significant number of tumors in adult Sencar. When epidermal cells from Sencar and Balb/c mice were grown under low calcium conditions for 6 weeks without treatment, spontaneously altered foci were selected by a switch to higher calcium. Sencar cultures had 13 fold more spontaneous colonies than Balb/c cultures. Likewise when Sencar cells were cloned as primary cultures, colony size at 2 weeks was 10 fold greater than Balb/c cells cloned at the same time. These results suggest that Sencar mice have inherent changes in the biological behavior of their keratinocytes, and such changes may be associated with the increased susceptibility of these cells to chemical carcinogenesis.

The interaction of carcinogens with DNA has been studied by a unique methodology pioneered by this Section. Antibodies have been developed in rabbits against N-(8)guanyl-2-acetylaminofluorene (G-8-AAF) and N-(8)-guanyl-2-aminofluorene (G-8-AF) the major guanosine adducts formed in vivo and in vitro by the interaction of nucleic acids with the aromatic amine carcinogen 2-acetylaminofluorene (AAF). The antisera obtained have been utilized to detect picomoles of each C-8 adduct in a competition radioimmunoassay utilizing ^{3}H-G-8-AAF or ^{3}H-G-8-AF. A linear regression equation has been established by computer techniques which allows precise quantitation of each C-8 adduct in an unknown mixture in which dG-8-AAF comprises less than 10% of adduct; for mixtures with more than 10% dG-8-AAF, proportions can be estimated by comparison with standard mixtures. A variety of cultured cell types exposed to N-acetoxyacetylaminofluorene have been surveyed to determine the innate capacity of each type to deacetylate the carcinogen during the process of adduct formation.

The C-8 adduct in most cell types studied (mouse and rat keratinocytes and fibroblasts, human fibroblasts) was >90% deacetylated (dG-8-AF). Rat hepatocytes in primary culture were exceptional in that they contained >80% of adduct as acetylated dG-8-AAF. The acetylated form was also the major product when cultured hepatocytes were exposed to AAF. However, DNA from livers of rats fed or injected with AAF contains a sizeable portion of the C-8 adduct as deacetylated, suggesting that cultured hepatocytes may not be performing the same metabolic steps as rat liver in vivo. In mouse keratinocyte cultures, conditions have been sought to vary the relative formation of acetylated and deacetylated DNA adducts to determine the biological importance of each. Exposure to N-Ac-AAF during growth in low calcium medium or in concert with the drugs ethidium bromide, harman or sodium butyrate were without effect. Exposure in serum free medium enhanced total C-8 binding by 2-5 fold and increased the acetylated adduct only by 4-5%. Exposure in concert with the microsomal deacetylase inhibitor paraoxon (10^{-8} - 10^{-5}M) dramatically reduced total binding to 1% of control and all of the adduct which did form was dG-8-AAF. The paraoxon effect occurred in the absence of cytotoxicity and provides a way to manipulate the proportion of adducts formed. In collaboration with the Human Tissue Studies Section, we have increased the sensitivity of the immunological detection of AAF adducts by 60 fold through the use of USERIA assay. This assay can still distinguish AAF and AF substitution and brings the level of detection of adducts to the range of a few femtomoles.

Antisera have also been developed against DNA substituted with the 7,8-diol, 9,10 epoxide of benzo[a]pyrene (BPDE I, the anti-isomer). The antigen was synthesized to yield >99% of adducts as trans(7R)-(benzo[a]pyrene)-N^2deoxyguanosine (BPdG). This antiserum has a higher affinity for BP substituted DNA than for the isolated BP-dG adduct suggesting antibody recognition of the DNA backbone. The antisera do not cross-react with the carcinogen or nucleoside separately. The affinity of these BP-DNA antibodies is greater for substituted denatured DNA than for substituted native DNA. An RIA developed with this antiserum detects binding in epidermal cells exposed to either benzo[a]pyrene (BP) or BPDE I. Since >90% of the BP adducts in epidermal cells in vivo or in vitro are G adducts as determined by high pressure liquid chromatography, this RIA is a valid measure of most of the DNA binding produced by BP and derivatives. During 24 hours after exposure to BPDE, approximately 40% of the guanosine adducts are removed as detected by RIA. Indirect immunofluorescence of epidermal cells exposed to BPDE yields intense nuclear fluorescence when the BPdG antiserum is used, but no fluorescence with preimmune serum.

Tumor promotion by phorbol esters has been an area of intense study in this laboratory. In the last year we have focused on the events associated with the initial responses of epidermal cells exposed to phorbol esters and particularly 12-O-tetradecanoylphorbol-13-acetate (TPA). Other studies are directed toward understanding the effect of this interaction on

cellular function and the mechanism by which antipromoters interfere in this process. Phorbol ester induced increase in activity of the enzyme ornithine decarboxylase (ODC) has been a reliable early marker for promoter effects in epidermis. While it is known that this induction requires transcription and translation, the mechanism of ODC gene activation is not understood. For this reason, agents which induce more defined genomic alterations have been studied for their effect on ODC induction alone and in concert with TPA. Ultraviolet light (10 J/m^2) (UVL) induces ODC with biphasic kinetics and peak inductions at 4 hours and 15 hours after exposure. At lower doses (2-5 J/m^2) UVL enhances ODC induction by TPA and at higher doses (20 J/m^2) it suppresses TPA-induced ODC. The inducing and enhancing properties of UVL can be separated by appropriate culture conditions. Mitomycin C produces DNA strand breaks and DNA-protein crosslinks. It induces ODC in a biphasic pattern similar to UVL but has no enhancing properties on TPA induction. Hydrogen peroxide damages DNA by a free radical mechanism and induces ODC with a peak at 4 hours after exposure. It does not enhance TPA induction. Thus DNA damaging agents induce the enzyme in a manner similar to UVL but do not have the enhancing properties of UVL. Caffeine, which blocks the closure of single strand gaps in DNA, does not induce ODC but enhances the induction by TPA. However, caffeine produces other cellular changes which must be considered prior to concluding that maintenance of single strand gaps could account for the enhancing properties of caffeine. Whether DNA damage at the site of the ODC gene is critical for its activation and whether TPA can produce this damage remains to be determined. Inhibitors of TPA induced ODC activity and tumor promotion are useful as probes to study both the mechanism of inhibition and the critical events required for each phenomenon. In the past year, several new inhibitors have been identified and studied. Local anesthetics are potent inhibitors of ODC induction by TPA and UVL. They appear to act by a mechanism similar to the inhibition produced by putrescine, the product of ODC activity. Lidocaine, like putrescine, probably acts by inhibiting the translation of the enzyme or by stimulation of an antizyme. Preliminary data suggest that local anesthetics inhibit tumor promotion on mouse skin. Ammonium chloride is an inhibitor of some receptor mediated processes and is a potent inhibitor of TPA induced ODC in vitro. Its action on promotion has not been tested.

The availability of inducers of ODC with partially distinct mechanisms of action (UVL and TPA) has provided a way to study the site of action of a variety of agents which alter the induction. Our data suggested that the early portion of the induction pathways differ for UVL and TPA while the transcription and translation portions are probably common. Retinoids are more potent inhibitors of TPA induction than UVL induction and thus probably inhibit an early event. This is supported by the observation that retinoid exposure prior to TPA is more effective than after TPA while retinoic acid still inhibits ODC induction by UV

when added as late as 4 hours after irradiation. Local anesthetics and the protease inhibitor TLCK inhibit both inductions equally and probably act on the common pathway. The steroid fluocinolone acetonide enhances both activities to the same extent and probably acts on the common pathway also.

The capacity to study cells in the basal or differentiating population simply by manipulating extracellular calcium concentration provided a means to examine TPA responsiveness of subpopulations of keratinocytes. Under low calcium conditions (basal cells) ODC induction by TPA is enhanced up to 30 fold probably reflecting a larger proportion of responsive cells. Cells remain responsive for many weeks in culture under these conditions. However, induction of ODC by UVL is very poor in low calcium medium although the enhancing activity for TPA induction is maintained. Thus the inducing and enhancing properties of UVL can be distinguished by these culture conditions. When low calcium cells are induced to differentiate by switch to high calcium, responsiveness to TPA induction of ODC is lost within 6 hours. Within 2 hours up to 50% of responsiveness is lost. Thus epidermal differentiation is associated with a loss of TPA responsiveness.

During the past year several new TPA induced cellular changes have been studied. Our early studies had indicated that under standard culture conditions, TPA exposure resulted in a decrease in epidermal transglutaminase (TG) over a 72 hour culture period. This was associated with a loss of differentiated cells and a selection of a proliferative population. However, when the basal cell population (in low calcium) is exposed to TPA, there is an induction of transglutaminase activity which peaks at 10-12 hours and gradually decays over 24 hours. This induction is dependent on transcription and translation and dose of TPA. TG induction may indicate that TPA stimulates epidermal differentiation. Alternatively the enzyme may be involved in other cellular reactions, perhaps related to gene activation. The activation of new protein synthesis in response to TPA has also been studied in collaboration with Dr. Michael Gottesman of the Laboratory of Molecular Biology of the National Cancer Institute. Within 6 hours of TPA treatment of mouse skin in vivo or mouse keratinocytes in vitro, there is a large (3-10 fold) increase in the synthesis of a major secretory glycoprotein called MEP. The synthesis of this protein, MW 35K, had previously been shown to be elevated in transformed but not normal fibroblasts. A function for this protein has not been established. Only phorbol esters with promoting activity induce this increase in MEP synthesis. Within several hours of TPA exposure in vitro, increased or new synthesis of 6 epidermal proteins has been identified by 2 dimensional gel electrophoresis. Many of these proteins are no longer observed by 24 hours after exposure. Non-promoting phorbol esters do not induce these proteins. Identification of these unique proteins and correlation with tumor promotion will be the objective of future experiments.

Significance to Biomedical Research and the Program of the Institute: The majority of human cancers are associated with environmental exposures and most of the tumors are of epithelial origin. Animal models have been extremely useful for bioassay and some mechanistic studies, but they are not as useful for investigations at the cellular level because of complicated interactions between host and environment as well as physiological variations within an experimental protocol. The use of cell culture systems, particularly those of epithelial origin, offers the opportunity to extend the present conceptual models of carcinogenesis mechanisms to a more basic and cellular level. In addition, cell cultures ultimately should be useful and inexpensive as bioassay screening procedures to detect carcinogens and cocarcinogens in the environment. Many of our present basic concepts concerning the pathogenesis of cancer were developed from studies utilizing carcinogen painting on mouse skin. The irreversibility of initiation, the phenomena of cocarcinogenesis and tumor promotion, the role of hyperplasia and metabolism are examples of such concepts. The development of a cell culture system for epidermal carcinogenesis has been a major advance toward extending our knowledge of mechanisms of carcinogenesis. Earlier efforts were directed to proving that epidermal cells in culture responded to carcinogens and promoting agents as in vivo. In almost every parameter studied this was the case. Differentiation, metabolism, proliferation, activation and binding of carcinogens, and promoter responses were highly analogous to the in vivo situation. These findings enhanced the validity of any subsequent observations made in vitro. For the last several years the model system has been utilized to a much greater degree to ask questions about mechanisms of transformation, carcinogen and promoter interactions, and the role of anticarcinogenic agents such as retinoids and steroids. The routine isolation of cells resistant to selection for terminal differentiation as well as the development of nontumorigenic and tumorigenic cell strains offers the opportunity to study the cancer phenotype and tumor progression. The role of differentiation in carcinogenesis and the mechanism by which promoting agents induce progressive biological changes can also be studied. Antigenic markers have become useful tools to detect normal and abnormal states and to provide a rapid assay for differentiation. The use of cells from sensitive and resistant animals offers the opportunity to determine factors responsible for susceptibility. Greater understanding of mechanisms of tumor promotion and the stages of tumor progression provides an opportunity to devise schemes for intervention in the process of carcinogenesis prior to the development of overt malignancy. Evidence for such a possibility is already apparent from studies of steroid hormones, retinoids and local anesthetics.

The development of an immunoassay for carcinogen-DNA adducts should have a number of important uses such as detection of carcinogens in human populations, following the removal of carcinogen from DNA and localizing the site of binding by a combination of immunological and morphological techniques. Finally while bridging the gap between relevant animal models and in vitro systems, this laboratory is simultaneously developing the analogous human tissue model in vitro. Thus,

ultimately a chain of systems will be available to determine the relevance of findings in any one model for the entire spectrum of models including the human.

Proposed Course of Project: Efforts during the previous year have markedly improved the in vitro model for epidermal carcinogenesis. Future studies will be directed to optimizing the model as a transformation assay. Both mass culture and clonal assays in which the Ca^{++} levels in culture medium will be used to select for cells with altered differentiation after carcinogen treatment, will be firmly established. Since malignant transformation is often (if not always) accompanied by altered differentiation, there is a reasonable probability that this change in differentiation will reflect a response which is associated with or parallel to that of malignant change and can be quantitated as a short term assay. The progression from altered differentiation to malignant transformation will then be studied with particular emphasis on the role of tumor promoters in this progression. Mutagenesis assays will be established with epidermal cell lines selected for high cloning efficiency. Similar models using human keratinocytes will be developed in parallel with these advances in the murine system. We will increase our efforts to isolate and characterize initiated cells both from adult mouse skin and in vitro assays. Present results suggest that initiated cells have an enhanced potential for in vitro growth. Using mice initiated in vivo, we will attempt to clone initiated cells or to select against normal cells in the starting population. Resistance to terminal differentiation will be used as the selection process. Characterization of initiated cells will emphasize the search for specific markers (biological, histochemical, enzymatic, immunological) of the initiated state and for unique responses to tumor promoters.

New in vitro markers for epidermal preneoplasia or neoplasia will be sought. Changes in epidermal transglutaminase or gamma glutamyltranspeptidase will be assayed in a series of normal, premalignant and malignant cells. The presence of new filamentous proteins such as the mesenchymal 10 nm filament, reflecting aberrant expression associated with neoplasia, will be explored by gel electrophoresis and immunofluorescence of transformed epidermal cells. Acquisition of other markers such as growth in ultralow calcium medium (< 0.02 mM) or secretion of new proteins (such as MEP) will be assessed. If a unique protein is found, antibodies will be produced and an RIA will be developed. This could provide a sensitive monitor for early changes induced by carcinogens in vitro. Benign and malignant tumors derived in vivo will be studied in a similar way to confirm in vitro results.

Studies to elucidate the mechanism of differential susceptibility to skin carcinogenesis in Sencar and Balb/c mice will continue. Differences in DNA repair capacity, DNA polymerase activity, evidence for endogenous virus content and endogenous growth factor production will be assessed. The suggestion that initiated cells preexist in Sencar mice will be further

explored utilizing markers for preneoplasia when developed and by additional in vitro and in vivo studies in which TPA exposure is withdrawn at various times. The use of a skin graft system on nude mice is also in progress to establish if sensitivity resides in the host or target tissue.

The remarkable sensitivity and specificity of antibodies raised against carcinogen-DNA adducts provides a new approach to bioassay and mechanistic studies. As an applied methodology, this technique should allow for detection of persistent adducts in cells and tissues from human populations exposed to environmental carcinogens such as benzo[a]pyrene. The collection and assay of such material is currently being coordinated with Dr. I. B. Weinstein of Columbia University. During the past year, we have succeeded in identifying conditions to alter the ratio of acetylated and deacetylated adducts obtained after exposure of epidermal cells to AAF. The deacetylated derivative is reportedly more mutagenic in bioassays. During the next year, we will examine the effect of varying the relative ratio of each of these derivatives in the newly developed epidermal carcinogenesis and mutagenesis assays. In addition the relative proportions and persistence of AAF adducts will be compared in normal and altered liver foci from rats fed AAF in vivo. The production of antibodies to the dG-N^2-AAF derivative will be attempted by hybridoma technology and other monoclonal antibodies will be prepared. Collaborative studies are presently underway utilizing electron microscopic immunohistochemistry (ferritin labeling) to detect specific localization of AAF binding to selective portions of DNA. In particular, binding to initiation sites in replicons is being examined after isolation of these sites by density gradient centrifugation. Similar studies will be attempted with the antibody to BPDEI-DNA. We will also use this technique to determine if gaps in nascent DNA after exposure of cells to BPDE are localized opposite adducts. Immunofluorescence techniques will be applied to study subcellular localization, persistence and tissue distribution of bound carcinogen.

Efforts to elucidate the mechanism of action of phorbol ester tumor promoters will be continued. The early effects of promoters will be monitored using ODC, transglutaminase and other markers as an assay. Studies to define coordinate expression among various responses will be conducted to gain an understanding of the sequential relationship of early responses. The presence of specific phorbol ester receptors on the cell surface will be studied in collaboration with Dr. Peter Blumberg of Harvard University. In particular, alterations in receptor level produced by retinoids or by the induction of terminal differentiation will be studied and correlated to change in function. To facilitate the isolation of a receptor, antibodies will be produced against TPA. The mechanism of action of antipromoter compounds will also be a continuing area of investigation. Utilizing ultraviolet light induced by ODC as a control stimulus, the specific action of retinoids, steroids and other inhibitors of the TPA response will be sought. We will attempt to determine which metabolic processes are required for the action of anti-promoters by

simultaneous exposure of cells to metabolic inhibitors and inhibitors of tumor promotion. Studies to define the nature and specific function of the new proteins induced by TPA in mouse skin will be pursued. Also we will expand our studies to determine the mechanism by which phorbol esters activate the transcription of specific genes. As has been our custom, all new information gathered from in vitro models will be confirmed by in vivo experimentation when possible.

Studies on the effects of carcinogens and tumor promoters on specific cellular functions have been facilitated by the development of laboratory methods for the monitoring of epidermal differentiation and keratin protein synthesis. Preliminary evidence for the in vitro translation of keratin protein suggests that the isolation of the RNA message for this product is feasible. Such an accomplishment would then allow the synthesis of the keratin genes by reverse transcription. Utilizing this approach, in combination with our sensitive immunological probes for carcinogen binding, one could monitor the effects of carcinogens (and tumor promoters) on a specific gene sequence. In particular the effects of carcinogen binding on transcription of a specific gene could be followed. Changes in the number of copies of this gene in normal (regulated differentiation), papilloma (accentuated differentiation), and carcinoma (diminished terminal differentiation) cells could be assessed. Gene rearrangements in such cells or in cells treated by initiators or promoters could be monitored by two dimensional gel electrophoresis of epidermal DNA subjected to restriction endonucleases. The availability of specific genetic probes would provide a direct approach to study the effects of carcinogenic agents on the regulation of genetic activity of the cell.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04513-05 LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Metabolism of Chemical Carcinogens by Cultured Esophagus, Bronchus and Pancreatic Duct from Humans and Experimental Animals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human bronchus, pancreatic duct and esophagus can be cultured for several weeks in a chemically-defined medium. This controlled experimental setting provides an excellent in vitro system to study the metabolism of chemical carcinogens including those found in tobacco smoke and the environment. Several classes of chemical carcinogens, polynuclear aromatic hydrocarbons, N-nitrosamines, hydrazines, aromatic amines and mycotoxins can be metabolically activated by human tissues.

The metabolic pathways leading to the formation of DNA adducts in cultured bronchus and esophagus have been defined by benzo[a]pyrene, 7, 12-dimethylbenz[a]-anthracene, aflatoxin B_1, and N-nitrosodimethylamine. The pathways to organic-extractable and water-soluble metabolites of benzo[a]pyrene were qualitatively similar in all 3 tissues. The adducts between these carcinogens and DNA in human bronchus and esophagus are essentially the same as those found in experimental animals in which the chemicals are carcinogenic. Interindividual differences in carcinogen-DNA binding values vary 50- to 150-fold. A quantitative variation in carcinogen binding values in different target tissues within an individual was also found.

Objectives: To determine the metabolic pathways of chemical carcinogens in target tissues of experimental animals and humans. To measure inter-individual and inter-tissue variations in the metabolism of carcinogens.

Methods Employed: Explant culture of human and animal tissues; quantitative high-resolution light microscopic autoradiography; isolation of cellular macromolecules; high pressure liquid chromatography; enzyme assays.

Major Findings: Cultured human bronchial mucosa can metabolically activate procarcinogens [polynuclear aromatic hydrocarbons: 7,12-dimethylbenz-[a]anthracene (DMBA), 3-methylcholanthrene (MCA), benzo[a]pyrene (BP) and dibenz[a,h]anthracene (DBA); N-nitrosamines: N-nitrosodimethylamine (DMN), N-nitrosodiethylamine (DEN), N-nitrosopiperidine (NPd), N-nitrosopyrrolidine (NPy) and N,N'-dinitrosopiperazine (DNP); a substituted hydrazine: 1,2-dimethylhydrazine (1,2-(DMH); a mycotoxin: aflatoxin B_1 (AFB_1); and an aromatic amine, 2-aminoacetylfluorene] into forms that bind to celular macromolecules including DNA.

The metabolism of BP has been extensively studied in explants of human bronchus cultured in a chemically defined medium. When compared to BP, binding values of its weakly carcinogenic analog, benzo[e]pyrene, were more than 100-fold less. Results from the trans addition of the 2-amino group of guanine to the 10 position of (+)7R,8S,9R,10R enantiomer of BP-7,8-dihydrodiol-9,10-oxide (BP diol epoxide I).

In a combined epidemiology-laboratory study we are comparing the metabolism of benzo[a]pyrene (BP) in cultured non-tumorous bronchial mucosa from cancer and non-cancer patients. Patient history and clinical findings are obtained from interviews and hospital records. Lung cancers are classified by cytochemistry and both light and electron microscopy. Macroscopically normal bronchial explants, collected at time of surgery or immediate autopsy, are maintained in chemically-defined medium for 7 days. BP (1.5 μM) is then added for 24 hr. Binding levels of BP to mucosal DNA (pmoles per 10 mg DNA) are being measured as part of this study. In the initial 79 patients (21 without and 58 with lung cancer), the interindividual variation among samples from single cases is less than 2-fold. When compared to binding levels in non-cancer patients, (18, mean; 2-82, range) higher levels are found in bronchi from patients with (a) epidermoid differentiated carcinomas including combined epidermoid-adenocarcinoma with a well-differentiated epidermoid component (34; 2-111; $p < 0.01$) or (b) mucous differentiated cancers with a non-glandular pattern (35; 11-96; $p < 0.025$), while no significant differences are found in bronchi from patients with glandular, mucous differentiated cancers (15; 1-45). Patient age, sex and history of tobacco smoking and consumption of alcoholic beverages are not confounding factors for the associations between binding levels and tumor types.

The extrapolation of carcinogenesis data among animal species depends in part on qualitative and quantitative differences between metabolic acti-

vation and deactivation of procarcinogens. Therefore, the metabolism of benzo[a]pyrene (BP) by cultured tracheobronchial tissues from different species - human, bovine, hamster, rat and mouse - has been investigated. The total metabolism, as measured by both organic solvent-extractable and water-soluble metabolites of BP, was substantial in the respiratory tract from humans and from animal species susceptible to the carcinogenic action of BP. The ratio of organic extractable metabolites to water-soluble metabolites was greater than one in hamster, human and C57Bl/6N mouse, but less than one in rat, bovine and DBA/2N mouse, suggesting that determination of both activation and deactivation pathways are important in assessing carcinogenic risk of a chemical. Sulphate esters and glutathione conjugates were the major water-soluble metabolites in all animal species; tetrols and diols were the major organic extractable metabolites. The level of trans-7,8-dihydro-7,8-dihydroxybenzo[a]pyrene, the proximate carcinogenic form of BP, was three times higher in C57Bl/6N, than in DBA/2N mouse trachea. Trans-9,10-dihydro-9,10-dihydroxybenzo[a]pyrene was the major metabolite formed by cultured hamster trachea. The binding levels of BP to cellular DNA were quite similar in all tissues, although slightly higher binding was observed in hamster trachea. Wide inter-individual variation in the binding of BP to DNA was seen in tissues from outbred species. The major BP-DNA adducts in all animal species were formed by interaction of benzo[a]pyrene diol-epoxide with the 2-amino group of deoxyguanosine. Both stereoisomeric forms of (±)-(7β,8α)-dihydroxy-(9α,10α)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE I) reacted with deoxyguanosine, the (7R)-form being the most reactive. No difference in the relative distribution of the various adducts was seen between the species except in the CD rat, where BPDE-deoxyadenosine adducts accounted for 20% of the total modification. In cultured hamster trachea the persistence of the different adducts was similar. In conclusion, the metabolism of BP is qualitatively similar in tracheobronchial tissues from both humans and animal species in which BP has been experimentally shown to be carcinogenic.

The 300-fold variation in the worldwide incidence of esophageal carcinoma suggests that environmental agents, including chemicals, cause this cancer. Since the interaction between chemical procarcinogens and human esophagus has not been previously studied, we examined the metabolic fate of BP, DMN and NPy in cultured non-tumorous esophagus from 2 patients with and 6 without esophageal carcinoma. Esophageal explants were cultured in a chemically-defined medium for 7 days prior to adding [^{3}H]BP (1.5 μM), [^{14}C]NPy (100 μM) or [^{14}C]DMN (100 μM) for 24 hrs. Radioactivity was found bound to protein (BP, DMN, NPy) and to DNA (BP, DMN). The major carcinogen-DNA adducts were: 1) trans addition of (+) BP diol epoxide I at the 10 position to the 2-amino of guanine; and 2) with DMN, N-7 methylguanine and O-6 methylguanine (O-6 MeG/N-7 MeG=0.3). The inter-individual variations in binding levels to DNA were 99-fold for BP and 10-fold for DMN. A positive correlation between DMN and BP binding to DNA was observed. In the 2 cases studied, the variation in binding levels among the 3 major anatomical segments (proximal, mid, distal) was less than 2-fold. The metabolism of BP into water-soluble metabolites varied among the 8 patients from 1 to 68% of the total metabolism. The quali-

tative chromatographic patterns of water-soluble metabolites [sulphate esters (range, 21-55%), glucuronide conjugates (7-37%), glutathione conjugates (24-66%)] and of organic-extractable metabolites were similar in all patients. A model system for the study of carcinogenesis in the rat esophagus has been developed and the metabolism of a series of N-nitrosamine carcinogens is being investigated in rat esophagus. After incubation of the explants with C-14 labeled nitrosamines [N-nitrosobenzyl-methylamine (NBMA); DMN, NPy, N-nitrosoethyl-methylamine (MEN)] at a concentration of 100 μM for 24 hrs, the activation was determined by measuring ^{14}C-CO_2 formation and assaying the binding level to DNA and to protein. The highest binding levels to DNA were with NBMA, an organ-specific carcinogen for rat esophagus, and DMN. The oxidation of NBMA mainly takes place in the benzyl-group as shown by the formation of benzaldehyde. The alkylation by the methyl group was about 10-fold higher than by the benzyl group. High levels of CO_2 were formed from DEN and 2-[^{14}C]MEN, indicating predominant oxidation of the α-position. Both β- and α-oxidation were observed in NPy.

The effect of aminophylline on the metabolism of benzo[a]pyrene by cultured human bronchus has been investigated by measuring the formation of both water-soluble and organo-soluble metabolites of benzo[a]pyrene and the amount of benzo[a]pyrene diol epoxide bound to mucosal DNA. Coincubation of benzo[a]pyrene with aminophylline caused an increase in benzo[a]pyrene diol epoxide bound to DNA. In addition, an increase in the ratio of benzo[a]pyrene sulphate esters to glutathione conjugates of benzo[a]pyrene metabolites was found.

Significance to Biomedical Research and the Program of the Institute: Methodologies developed for and utilized in studies of carcinogenesis in experimental animals and cell cultures can be successfully extended to similar investigations in human cells and tissues. These investigations should eventually aid in identifying both chemical carcingens and host factors determining susceptibility. Finally, these studies provide a much needed link between studies in experimental animals and man.

Proposed Course of the Project: Identify endogenous and exogenous factors that alter the metabolism of environmental procarcinogens. To continue combined laboratory-epidemiology study of carcinogen metabolism. To use ultramicroassays of carcingen metabolism and carcinogen-DNA adducts so that metabolism and repair can be studied in biopsy specimens. Correlate metabolism of chemical carcinogens between different target tissues within a single individual. Compare metabolism of chemical carcinogens in target tissues and possible "detector" cells, i.e., monocytes and macrophages. To continue comparative studies of carcinogen metabolism in cultured human esophagus to that found in cultured rat esophagus. To study the genomic distribution and modification of carcinogen-DNA adducts as well as their enzymatic repair.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE. PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-CP-04567-07 LEP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Carcinogenesis Studies in Human Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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12	7	5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Model systems for studying carcinogenesis directly in human target tissues (bronchus, colon, breast, esophagus and pancreatic duct) have been developed to assess the: a) mechanisms of carcinogenesis in human cells; b) variation of carcinogenic susceptibility among individuals; and c) validity of the extrapolation of carcinogenesis data from experimental animals to the human situation. Investigations to date indicate that metabolic pathways and carcinogen-DNA adducts are generally qualitatively similar in both human tissues and tissues of experimental animals in which these chemicals are carcinogenic. Clonal growth and serial passage of human bronchial and esophageal epithelial cells has been achieved. These human cells are being used to study in vitro carcinogenesis, DNA repair, carcinogen metabolism and control of differentiation. Immunoassays that are 100- to 1,000-fold more sensitive than radioimmunoassay and hybridoma-produced monoclonal antibodies to carcinogen-DNA adducts have been developed. These developments provide unique opportunities to detect carcinogen-DNA adducts in human biopsy specimens and to measure the intra- and intergenomic distribution and repair of these adducts at the molecular level.

PHS-6040

Objectives: To develop experimental systems to study carcinogenesis in human target tissues under controlled conditions. Such systems involve 3 major facets: 1) collection of viable human tissues; 2) *in vitro* maintenance of epithelial cells in cell and organ cultures; and 3) xenotransplantation of human tissues and cells into immunodeficient animals.

Methods Employed: Human tissues are obtained at either surgery or "immediate" autopsy. The tissue is maintained in culture and xenotransplanted into athymic nude mice. These tissues are exposed to chemical carcinogens *in vitro* and/or as a xenograft. The morphological lesions caused by carcinogens are examined by light and electron microscopy. Binding of carcinogens to macromolecules in epithelial cells is measured by biochemical and autoradiographic methods, which were previously developed, in part, in studies of animal models.

Major Findings: The methodology for each of the three major technical facets of this program (collection of tissues, explant and cell culture and xenotransplantation) has been refined. The maintenance of human tissue (bronchus, colon, peripheral lung, breast, esophagus and pancreatic duct) and human epithelial cells (bronchus and esophagus) in controlled experimental settings has allowed a series of interlocking studies including: 1) the metabolism of chemical carcinogens by human tissues and cells; 2) mutagenesis in mammalian cells (V-79 Chinese hamster cells) mediated by human tissues and cells; 3) induction of epithelial lesions by chemical and physical carcinogens; 4) molecular interactions between ultimate carcinogens and DNA and the repair of the DNA lesions.

Several classes of chemical carcinogens were found to be metabolically activated into forms that bind to cellular DNA. The major adducts between DNA and several chemical carcinogens (benzo[a]pyrene, 7,12-dimethylbenz[a]-anthracene, aflatoxin B_1, N-nitrosodimethylamine and 1,2-dimethylhydrazine) have been isolated and identified. Interindividual differences (50- to 150-fold) in values of carcinogen-DNA adducts has been found. Within a single individual, variation among various tissues in binding levels of chemical carcinogens were found. The relationship between formation of carcinogen-DNA adducts and individual's oncogenic susceptibility to the carcinogen is as yet not known. These biochemical studies of carcinogen binding to DNA are being correlated with biological studies, e.g., human tissue mediated mutagenesis. Both benzo[a]pyrene and its proximate carcinogen, 7,8-diol, are enzymatically activated by human bronchus, macrophages and monocytes into metabolites that cause mutations in V-79 cells. Our studies showing possible interaction between pulmonary macrophages and the bronchial epithelium in the metabolic activation of inhaled carcinogens focuses attention on interactions between nontarget cells and target cells in the processing of chemical carcinogens in organs and in the entire organism. An important strategy in our studies is to conduct parallel studies in cultured tissues and cells from experimental animals. For example, interspecies studies of benzo[a]pyrene metabolism in cultured tracheobronchial tissues have shown (a) wider interindividual differences in outbred versus inbred animal species, and (b) metabolic pathways of benzo[a]pyrene activation and deactivation and benzo[a]pyrene-DNA adducts are qualitatively similar.

Ultrasensitive enzymatic radioimmunoassay (USERIA) has been invented. USERIA is 100- and 1,000-fold more sensitive than radioimmunoassay for the detection of antigens, e.g., viruses, toxins, hormones, products of differentiation, carcinogen-DNA adducts, etc., and of specific antibodies. The coupling of the USERIA approach and hybridoma-produced monoclonal antibodies to carcinogen-DNA adducts should provide new opportunities to investigate the intra- and intergenomic carcinogen-induced DNA lesions and to search for carcinogen-DNA adducts in clinical specimens.

Morphological, cytochemical and immunological methods to identify in situ and cultured normal and neoplastic human epithelial cells have been refined.

Normal human bronchial epithelial cells can now be cloned (15% clonal efficiency) and serially cultured for 35-40 cell populations. These human epithelial cells are being used to study in vitro carcinogenesis, carcinogen metabolism, DNA repair and cellular differentiation.

The interaction between asbestos and cultured bronchial epithelium has been examined. Amosite asbestos enhanced both the labeling index with ^{3}H-TdR and cellular atypia in the epithelium. The penetration and intracellular localization of asbestos has been visualized by scanning and high-voltage electron microscopy.

Studies in progress suggest that carcinogens cause basal cell hyperplasia and atypical squamous metaplasia in cultured and in xenotransplanted human bronchial epithelium. The biological fate of these preneoplastic lesions is being determined.

These investigations are conducted in collaboration with NIH contractors, University of Maryland, National Naval Medical Center, Litton Bionetics and Washington Veterans Administration Hospital. Surgical and autopsy specimens are provided to NCI and to Litton Bionetics by Washington Veterans Administration Hospital, National Naval Medical Center and University of Maryland. Methods to culture explants of human and experimental animal tissues were developed in parallel at the University of Maryland and the NCI. Methods to culture human epithelial cells were established at the NCI. Investigations concerning the metabolism of chemical carcinogens and the development of the human tissue mediated mutagenesis assay were conducted primarily at the NCI. Cultured tissues are exposed to carcinogens at the NCI and at the University of Maryland. Human tissues and cells are implanted into athymic nude mice at Litton Bionetics.

Significance to Biomedical Research and the Program of the Institute: The extrapolation of carcinogenesis data from studies utilizing experimental animals in man presents several complex problems. One approach to provide a link between these experimental data and human cancer is the development of an experimental system to study carcinogenesis in important human target tissues. Such a system could aid in the identification of individuals who are highly susceptible to environmental carcinogens, and methods of intervention could be tested in this experimental system prior to clinical trials in man.

Proposed Course of Project: Attempts are continually being made to improve each interlocking facet of this program. Studies have been initiated to develop epithelial cell cultures derived from human colon. These cultured cells will be characterized by morphological, immunological and biochemical methods. Attempts to transform human bronchial epithelial cells with chemical carcinogens will be made. Correlations will be made among the formation of carcinogen-DNA adducts in human tissues, mutations in V-79 cells mediated by human tissues and morphological lesions in cultured human epithelia caused by chemical carcinogens. Responses to chemical carcinogens in cultured human tissues will be compared to those found in human pathology and in animal models. Carcinogen-modified DNA will be further analyzed at the molecular level. The occurrence, persistence, repair and functional consequences of both direct and indirect modifications of DNA will be examined.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04798-10-LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Metabolism and Mode of Action of Vitamin A

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A reverse phase HPLC system was developed to separate retinylphosphate(RP) from other retinoids. Application of this technique to metabolic studies in mouse intestine has shown that the phosphorylated derivatives of retinol constitute 12% of the products at 3 hr and 42% at 24 hr after administration of [1-^{3}H]retinol. HPLC was also used to characterize the mannolipids obtained from retinol and retinoic acid in 3T12 cells. The mixture of the [15-^{3}H]retinol- and [15-^{14}C]retinoic acid-derived mannolipids was subjected to mild hydrolysis, after purification by HPLC. Hydrolysis studies demonstrate that 3T12 cells synthesize mannosylretinylphosphate (MRP) from retinol, and a mannosylretinoidphosphate (MXP) from retinoic acid. These results exclude the possibility of a reduction of retinoic acid to retinol, but suggest that a retinol-like compound formed from RA is incorporated into MXP. Two different enzymes were shown to be responsible for MRP and dolichyl-mannosylphosphate synthesis. [^{14}C]Mannosyl[^{3}H]retinyl[^{32}]phosphate was shown to donate [^{14}C]mannose to endogenous acceptors of rat liver membranes.

PHS-6040
(Rev. 10-76)

Objectives: 1. To develop suitable techniques for the measurement of phosphorylated metabolites of retinol and retinoic acid. 2. To study the function of retinol and retinoic acid or their metabolites as sugar carriers in biological membranes.

Methods Employed:

High-pressure liquid chromatography. High pressure liquid chromatography was performed on an Altex Model 322 MP programmable liquid chromatography system. The sensitivity of this system is dependent on the recorder, a linear Model 300 with a maximum AUFS of 0.01. The uv spectrophotometer was a Hitachi Model 100-30 equipped with a variable wavelength between 195 and 850 nm.

Fluorometer Model FS 970 from the Schoeffel Instrument Corporation (Westwood, N.J.) was also connected with the HPLC system, in series and before the spectrophotometer. The high-pressure liquid chromatography columns were obtained from Whatman Inc., Clifton, New Jersey. A Partisil-10-ODS column (4.6 mm internal diameter x 25 cm) was used. The column was eluted with acetonitrile/water (55/45) at an initial flow rate of 1.0 ml/min for the first 10 min to elute retinylphosphate, mannosylretinylphosphate and retinoic acid; 3.5 ml/min for the following 20 min to elute retinol, retinal, retinylacetate, and anhydroretinol. The solvent ratio was then changed to acetonitrile/water (98/2) and the flow rate was adjusted to 1.2 ml/min for an additional 15 min, to elute retinylpalmitate.

Ion-pair HPLC was developed to aid in the characterization of the retinol-like metabolite of retinoic acid found in mannosylretinoidphosphate. In this system a mixture of acetonitrile and phosphate buffer pH 7.2 (37/63) was used at an initial flow rate of 2.2 ml/min for 30 min to elute all-trans mannosylretinylphosphate, all-trans retinylphosphate, 13 cis and all-trans retinoic acid. At 30 min the ratio of acetonitrile to phosphate buffer was changed (54/46) and the flow rate was adjusted to 3.5 ml/min to elute 13-cis retinol, all-trans retinol, retinal, retinyl acetate and all-trans anhydroretinol. At 45 min the phosphate buffer was replaced with water and the ratio of acetonitrile to water was changed to (98/2) and flow rate was adjusted to 1.5 ml/min for additional 15 min to elute retinyl palmitate.

Major Findings:

Separation of naturally occurring retinoids including phosphorylated derivatives of vitamin A. The separation of naturally occurring retinoids was achieved by reverse phase HPLC on a microparticulate octadecylsilane (ODS) column eluted with acetonitrile/water mixtures. A typical separation was obtained from a mixture of 200-250 ng of each of the following standard retinoids: retinylphosphate, retinoic acid, retinal, retinol, retinylacetate, anhydroretinol, and retinylpalmitate, all in the all-trans configuration.

Ammonium acetate was not needed for baseline separation of the various retinoids, using this solvent system. Moreover, analyses conducted in the presence of 1% ammonium acetate gave a relatively broader peak of retinylphosphate, which was eluted in the area of retinol. Retinylphosphate and its mannosylated derivative are eluted identically in this system. However, the inclusion of 1% ammonium acetate allows separation of these two products.

Application of this technique to metabolic studies in mouse intestine has shown that the phosphorylated derivatives of retinol, constitute approximately 12% of the radioactive products at 3 hr and 42% at 24 hr after administration of [1-^{3}H]retinol by stomach tube.

The biosynthesis of a mannolipid containing a metabolite of retinoic acid by 3T12 mouse fibroblasts. 3T12 Cells were chosen because of the activity of both, retinol and retinoic acid, in increasing adhesion as well as glycosylation in these cells.

3T12 Cells and their microsomes synthesize mannosylretinylphosphate from retinol and retinylphosphate. Therefore, it was of interest to investigate whether retinoic acid might also form a mannosylretinoidphosphate (MXP): this would explain why the acid can replace the alcohol in adhesion and glycosylation. Radioactivity from (11,12^{3}H; carboxy ^{14}C]retinoic acid) was incorporated into a compound indistinguishable from authentic mannosylretinylphosphate by column, thin layer and HPLC techniques. The ratio of ^{3}H/^{14}C was identical to that of the starting RA, thus excluding the possibility that decarboxylation occurs prior to mannosylphosphoryl formation. This lipid was easily cleaved by mild acid as for MRP, but with different products: MRP yielded all-trans anhydroretinol after mild acid treatment, whereas MXP yielded a product slightly more polar than all-trans anhydroretinol.

In a double-label experiment using carbinol-^{3}H retinol and carboxy-^{14}C retinoic acid, 3T12 cells synthesized [^{3}H]MRP and [^{14}C]MXP. MXP represented about 40% of the total radioactivity from [15-^{14}C]retinoic acid. The two products were cleaved with mild acid in seconds. Therefore, we conclude that:

1. 3T12 cells synthesize mannosylretinylphosphate from retinol.

2. 3T12 cells synthesize a mannosylretinoidphosphate (MXP) from RA, which constitutes up to 40% of the total metabolites of RA at 40 hours incubation.

3. Both MRP and MXP are hydrolyzed by mild acid in a few seconds at room temperature. MRP yields mannosephosphate and all-trans-anhydroretinol. MXP yields mannose-phosphate and a product which is more polar than all-trans and 13-cis anhydroretinol.

Demonstration of two mannosyl-transferases for mannosylretinylphosphate (MRP) and dolichylmannosylphosphate (DMP) synthesis. The presence of two distinct mannosyltransferases responsible for the two lipid intermediates MRP and DMP was demonstrated. A whole membrane fraction from the lymphoma cell line Thy^+ was able to synthesize DMP but lacked the MRP synthesizing enzyme, thus demonstrating that two different enzymes are responsible for the synthesis of the two lipid intermediates. In another development the two enzyme activities were solubilized from liver microsomes by different concentrations of the detergent Triton X-100.

Transfer of [^{14}C]mannose from [^{14}C]mannosyl[^{3}H]retinyl[^{32}P]phosphate to endogenous acceptors of rat liver membranes. The triple label donor was prepared from synthetic [^{3}H]retinyl[^{32}P]phosphate and guanosinediphospho-[^{14}C]mannose in the presence of rat liver microsomal membranes. The donor was shown to donate only [^{14}C]mannose to endogenous macromolecular membrane acceptors.

Significance to Biomedical Research and the Program of the Institute. It is the aim of this project to investigate the mechanism(s) by which vitamin A functions in the body. Inasmuch as vitamin A and its derivatives, the retinoids, are active as preventive agents of epithelial cancer, such investigation may yield useful information on mechanisms whereby normal tissue-specific phenotypic expression is maintained by retinoids.

A substantial body of work has shown a biochemical involvement of vitamin A at the level of the biosynthesis of glycoproteins. The phosphorylated vitamin appears to function in mammalian membranes as a carrier of mannosyl residues destined to specific glycoproteins. Some glycoproteins mediate cell to cell recognition and adhesion (e.g. fibronectins); others have hormonal functions in tissue growth and development (e.g. the gonadotropins); others yet display epithelioprotective functions (e.g. secretory mucins); thus, it is reasonable to propose that the newly found involvement of the phosphorylated vitamin in glycoprotein biosynthesis may explain its effect on mucus secretion, adhesion and the maintenance of normal phenotypic expression.

Proposed course of project:

To determine whether the lack of the specific enzyme which synthesizes mannosylretinylphosphate in the lymphoma cell line Thy^+ is common to other cell lines. 2. To identify the metabolite of retinoic acid synthesized by 3T12 cells and incorporated into the mannosylretinoidphosphate of these cells. 3. To solubilize and purify the mannosyl transferase(s) which utilize retinylphosphate as a substrate in mannose transfer reactions.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05092-02-LEP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pathology of tumors and other lesions induced in experimental animals by prenatal or postnatal exposure to chemical carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Selected direct-acting and metabolism-dependent compounds are administered by various routes to non-pregnant and to precisely timed-pregnant rodents and non-human primates, and the treated animals and their offspring are followed for subsequent tumor development. Neoplasms which develop are classified by light and electron microscopy, histochemistry, transplantation, and cultivation in vitro. Dose/response relationships are precisely quantitated to establish age-dependent qualitative and quantitative differences in susceptibility to different types of carcinogens in different organ systems.

Objectives: To explore and define the varying susceptibilities of different organ systems in rodents and primates to direct-acting and metabolism dependent carcinogens during prenatal and postnatal development, and precisely to characterize neoplastic and selected non-neoplastic conditions by light and electron microscopy, histochemistry, transplantation, explantation to cell and organ culture, and by other procedures as required.

Methods Employed: Carcinogenic chemicals specifically selected or designed for a given study are purified after purchase, or synthesized de novo and characterized thoroughly by chromatographic and spectroscopic procedures. Radiolabeled compounds are similarly prepared as required. Carcinogens in precise dosages are administered to non-pregnant or exactly timed-pregnant rodent (rat, mouse) or non-human primate (patas or rhesus monkey) subjects, and the treated animals and their offspring carefully followed for tumor development. Surgical procedures are devised as necessary to modify the processing of carcinogens (e.g., bile duct cannulation) in studies on organotropism of carcinogens, or to remove single operable growths from primates in order to prolong life and allow further observation for additional tumor development. Selected neoplasms and fetal tissues are explanted to cell culture and to athymic (Nu/Nu) mice for further study of their properties. Tumors are carefully classified histogenetically by light and electron microscopy and by histochemistry.

Major Findings: Studies on carcinogenesis by ethylnitrosourea (ENU) and diethylnitrosamine (DEN) in the Old World monkey, Erythrocebus patas, have been continued and expanded. Additional cases of both mesenchymal and epithelial tumors were observed in the offspring of monkeys given ENU intravenously during pregnancy, especially when treatment was begun during the first month of gestation. These additional findings confirm the tentative conclusions previously drawn that, like rodents, this representative primate species is quantitatively more susceptible to the direct-acting alkylating agent ENU during prenatal life, with animals exposed in utero exhibiting a higher incidence of tumors after a shorter latency than juvenile or adult animals given the same dose directly.

Additional findings in the offspring of monkeys treated during pregnancy include the observation of tumors of lining epithelia, especially the intestine. Intestinal tumors have also been seen in adult animals given ENU intravenously; both polypoid adenomas and invasive mucinous carcinomas have developed in animals treated postnatally. In contrast, colonic tumors developing after transplacental exposure have been exclusively polypoid, with abundant stroma, mimicking the so called "juvenile" polyps of the human intestine. Although these tumors have grown large enough to cause death by intestinal obstruction, none have metastasized or invaded the tunica muscularis or even the stalk of the polyp itself. This benign behavior of colonic tumors in monkeys exposed prenatally is like that of other tumors of both epithelial and mesenchymal origin induced prenatally in the patas; only one tumor, a nephroblastoma, has metastasized (to the lungs), although a large proportion of all neoplasms seen in the offspring have caused death by virtue of their size and location. In contrast, epithelial and endothelial tumors induced in adult animals have

metastasized widely and early. This includes choriocarcinoma, previously reported in patas females given ENU while pregnant. Studies on choiriocarcinoma have continued with current emphasis on characterizing the morphology of tumor cells and the pathogenesis of this rapidly fatal neoplasm.

Through collaboration with investigators in the NINCDS, we have been able to extend our studies on the transplacental carcinogenic effects of ENU to a second primate species, the rhesus (Macacca mulatta). Tumors of the connective tissues have now been confirmed in several offspring of rhesus females given ENU during pregnancy at doses comparable to those used in the patas experiments.

Tumors are generally of the same kinds as have been seen in patas, and have occurred during the first post-natal year. No tumors have been seen in adult rhesus given ENU at comparable dosage levels. This result tends to confirm the conclusions drawn from the patas experiments, and demonstrates that these results may be of general applicability to primates including man, and are not peculiar or unique to the patas. In particular, the important conclusion of heightened fetal susceptibility is confirmed and extended.

A further finding during the past year in the offspring of patas given ENU during pregnancy has been the occurrence of primary tumors of the nervous system, including both malignant tumors of the cerebral meninges and primary neuroectodermal tumors, including one ependymoma and a ganglioglioma. No tumors originating in the nervous system have been seen in adult animals given ENU. This observation extends the studies in rodents which have identified the nervous system as in general a more susceptible target organ during development in all species in which transplacental carcinogenesis has been studied.

Extensions of studies on the transplacental effects of direct acting alkylating agents in additional strains of rats and mice have yielded the interesting observation that, in response to a single exposure to ENU toward the end of gestation, strain B10.A mice preferentially develop a high incidence of invasive adenocarcinomas of the large and small intestines. These animals also develop the primary peripheral lung tumors and primary liver cell tumors characteristic of most strains of mice exposed to transplacental carcinogens; the preferential development of tumors of the intestinal mucosa, a lining epithelium, provides additional support for the hypothesis that prenatal exposures to chemical carcinogens may be significant in the development of tumors of certain lining epithelia, which in man characteristically give rise to clinically detectable malignancies during adult life, possibly as a consequence of continued exposure not only to primary carcinogens, but to various forms of cocarcinogens and tumor promoters.

Studies have continued on the biological effects of the α-acetoxy derivatives of dimethylnitrosamine (DMN-OAc). In the course of an experiment originally designed to define optimal conditions for investigation of cell cycle dependence of hepatocellular carcinogenesis in rats, DMN-OAc or methylnitrosourea (MNU) were given to partially hepatectomized rats as a single injection into the portal vein. An unexpectedly large fraction of the MNU passed through the

liver remnants, inducing tumors in many distant organ systems in high incidence, including the kidneys, intestinal tract, testicular mesothelium, nervous system, and other sites. A completely unexpected finding in the MNU study was 29 odontogenic tumors in 134 treated F344 rats. These tumors appear to arise near the roots of the continuously erupting incisor teeth. Ninety percent were complex odontomas, consisting of well differentiated but haphazardly arranged cementum, dentin, enamel and pulp. Dentin comprised the bulk of the tumor masses. In contrast, the remaining tumors (3 cases) were largely ameloblastic. This chance finding contributes a useful new model for the induction of such tumors in high yield, which are of potential use in the developmental biology of neoplasia; complex odontomas are composed of tissues derived from two germ layers, dental ectoderm and mesoderm, which must cooperatively exert inductive influences on each other in order to differentiate sequentially to form the extracellular products, dentin and enamel. In contrast, DMN-OAc, although dependent on intracellular esterase for its activation to a reactive ultimate carcinogen, gave rise to a significant incidence of primary liver cell tumors and to few tumors in other organ systems, suggesting that it might be a useful agent for the study of cell cycle dependence of susceptibility to carcinogenesis in partially hepatectomized animals.

Significance to Biomedical Research and the Program of the Institute: Research on animal models of human childhood neoplasms should provide an insight into the types of causative agents and modes of exposures responsible for childhood cancer. It is to be expected that natural selection would tend to eliminate genotypes in the human population which predispose individuals to the development of fetal neoplasms before attaining reproductive age, yet the incidence of tumors in childhood is relatively constant. Epidemiological studies have pointed to the occurrence of childhood neoplasms in association with certain types of congenital malformation which are non-inherited, and suggest that environmental agents, alone or in combination, may play a role in the induction of such neoplasms. The inducibility of tumors very similar to the pediatric tumors of man by chemical carcinogens in laboratory rodents and primates further supports this view.

Most tumors induced transplacentally in rodents are of adult types and appear during adult life in individuals exposed in utero, resembling human experience with diethylstilbestrol. The ENU studies in patas monkeys have provided experimental data indicating that both adult and pediatric tumor types develop in at least one species of primate in response to carcinogenic exposure in utero, and suggest that chemical carcinogens may be involved in the prenatal genesis of pediatric and possibly certain adult types of tumors in man. The demonstration of the inducibility of uterine choriocarcinoma by chemical carcinogens, at low exposure levels, first reported last year, further illustrates the importance of preventing human exposure to carcinogenic chemicals during pregnancy in either the workplace or environment.

Carcinomas of the gastrointestinal tract and pancreas comprise the largest source of cancer morbidity in the adult population of the United States. The

discovery, both in a particular strain of mice and in a representative non-human primate, that intestinal tumors are among the results of prenatal exposure to direct acting chemical carcinogens, raises the possibility that tumors of this sort may result not only from postnatal exposure to low level carcinogens over a long period of time, but may develop from a transient and even a single exposure to a potent carcinogen during a particularly susceptible period of development. Factors modifying the biology and evolution of these tumors of lining epithelia should be studied in this context.

Proposed Course: Patas monkeys exposed to ENU or DEN either transplacentally or directly (after weaning) should continue to be observed for the development of tumors. In vivo studies should emphasize further refinement of definition of periods of maximal prenatal susceptibility to direct-acting vs. enzyme-activated transplacental carcinogens, and the extent to which the enzymes which activate different classes of metabolism-dependent carcinogen can be induced by either the carcinogens or other agents in fetal, maternal and placental tissues at different stages of prenatal development. The possibility that the phenomenon of tumor promotion can be demonstrated in primates prenatally exposed to ENU, either by increasing the incidence of hepatocellular tumors by postnatal exposure to phenobarbital, DDT, etc. or by increasing the incidence of skin tumors by topical application of phorbol esters, will be explored. Pharmacodynamic studies will be continued to further study maternal-fetal distribution and tissue/organ localization of ENU-^{14}C in this species in comparison with rats and mice.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE. PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-CP-05093-02-LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Differentiation of fetal cells and tissues: modulating factors in organ-specific carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. M. Rice	Section Chief	LEP, NCI
Other:	J. Berman	Staff Fellow	LEP, NCI
	W. Edwards	Visiting Associate	LEP, NCI
	P. Donovan	Chemist	LEP, NCI
	A. Perantoni	Microbiologist	LEP, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The roles of morphogenetic differentiation in controlling the phenotypic expression of neoplastic transformation, the degree of malignancy of tumors, and the susceptibility of developing organs to carcinogenesis are studied using organ culture and tissue transplantation techniques, with current emphasis on the kidney.

PHS-6040

Objectives: To identify and characterize those aspects of morphogenetic differentiation which modify the consequences of prenatal exposure to chemical carcinogens, especially in the nervous and genitourinary systems. To modify the biological behavior of chemically induced tumors, including their rate of growth, capacity to invade, and ability to metastasize. The ultimate objective is to elucidate the control of expression of the neoplastic phenotype in transformed cells.

Methods employed: Short and long-term cell and organ culture techniques are developed, the features of tissue rudiments maintained therein characterized by histochemical, light microscopic, and ultrastructural techniques. Cultures of both normal fetal organ rudiments and selected tumors are utilized to explore the effects of morphogenetic differentiation and its induction on the behavior of tumors of undifferentiated cell type, including nephroblastic tumors of the kidney. Tumors are induced transplacentally or by direct treatment in experimental animals to provide suitable material for study, and are transplanted serially in appropriate recipients to develop standard, manipulatable models for studies in vitro. Surgical procedures relating to tissue transplantation are adapted as necessary to study the capacity of various recipient sites to modify differentiation of selected transplantable tumors. Substances and tissues known to influence differentiation are combined with tumors and normal undifferentiated organ rudiments in organ culture and their effects on differentiation of normal and neoplastic tissues characterized. All studies are performed in more than one species, selecting species (such as the rat and mouse) which can be readily manipulated in the laboratory and in which responses of selected organ systems, such as the kidney and brain, to chemical carcinogens during fetal life vary extremely both in quantitative responses to chemical carcinogens and in the nature of tumors induced.

Major findings: Previous reports have described our development of procedures for culturing fetal organ rudiments and for producing primary and established monolayer cultures of fetal and adult epithelial organs. Rudimentary fetal organs have been found to dissociate readily in the presence of a chelating agent (EDTA), and in the absence of proteolytic agents, in contrast to adult organs where proteolytic digestion is required for tissue dissociation and successful culture. Rudimentary kidney, characterized by electron microscopy, is essentially devoid of desmosome structures. The role of desmosomes in expression of the epithelial phenotype in vivo and in cell culture is currently being explored.

Efforts to manipulate morphology in differentiation of normal and neoplastic kidney tissues by modification of culture conditions continues. Under current organ culture conditions, where kidney rudiments are grown on gelfoam sponge on a rocker assembly in five per cent CO_2 and atmospheric O_2 in media supplemented with the hormones hydrocortisone and insulin, fetal kidney explants survive and continue to differentiate for at least 9-12, and occasionally as long as 20, days. This is an improvement over previous efforts in which a maximum of 7 days survival without necrosis was achieved. The greater viability and longer survival of fetal kidney rudiments in recent studies may reflect

improved culture techniques and may also reflect a greater capacity to support growth and differentiation by different lots of fetal bovine serum.

Undifferentiated (blastemal cell) tumors induced by the carcinogen methyl-(methoxymethyl)nitrosamine in newborn F344 male and female rats have been successfully transplanted to syngeneic recipients, and of the 27 primary tumors initially transplanted, 3 of the 16 that grew intradermally have evolved into noninvasive, rapidly growing and completely undifferentiated tumors which strongly resemble undifferentiated renal blastem in their morphologic features. They are devoid of any evidence of epithelial differentiation by histochemistry and light microscopy; ultrastructural characterization of these lines is in progress. Fragments from transplant tumors, including the three permanent lines, have been studied in organ culture, generally with maintenance of their highly cellular, undifferentiated pattern of growth. Under certain circumstances, these tumors have occasionally and without additional manipulation differentiated to form structures in organ culture indistinguishable from those seen during early tubulogenesis in the permanent kidney of the fetal rat. This finding suggests that the experimental materials are now at hand for systematic study of factors influencing differentiation of normal and neoplastic renal blastem originating from the rat, and for comparing it to comparable tissues in the mouse in which differentiation, as judged from the types of tumors seen, is much more efficient.

In order to ascertain that the selected culture conditions would allow full expression of differentiated renal cell function, permanent cell lines of human Wilms' tumor origin (TuWi) and from adult porcine kidney have been characterized and their media requirements determined. It has been found that enzymes characteristic of adult renal epithelium, including gamma glutamyltranspeptidase (GGT), can readily be demonstrated histochemically and quantitated biochemically. Accordingly, the methodology is available for characterizing any induced differentiation in organ cultures or cell cultures of blastemal-cell tumors.

Significance to biomedical research and the program of the Institute: Studies in rodents have shown that a fetus may be as much as two orders of magnitude more susceptible to carcinogens than an adult of the same species, strain, and sex. The precise reasons for this enhanced vulnerability are not clearly understood, and the fact remains unexplained that, in rodents, carcinogens acting on differentiating fetal tissues induce principally tumors of adult epithelial morphology. Many tumors that develop as a consequence of transplacental exposure to carcinogens are morphologically identical to those inducible in adults. In the mouse kidney, for example, only adenomas and a few carcinomas originating from proximal convoluted tubules develop after prenatal exposure to carcinogens, when the kidney is mostly undifferentiated. This suggests that the fundamental genetic damage inflicted on undifferentiated fetal cells does not preclude subsequent programmed differentiation. The fact that differentiation overrides expression of neoplastic transformation in a given organ system (e.g., the kidney) of certain species such as the mouse, but does not do so in others, such as the rat,

provides a route to exploration of the basic nature of cellular differentiation in the control of neoplastic growth, in the context of prenatal susceptibility to carcinogens.

Proposed course: Rat renal "blastemal-cell" tumors will continue to be studied in transplantation and in cell and organ culture to determine whether the morphologically undifferentiated tumors can be induced to form characteristic epithelial structures resembling renal tubules, and whether the enzymes characteristic of renal epithelium will develop as markers of morphologically demonstrable differentiation. In organ culture, both natural (ureteric bud) and heterotopic (fetal spinal cord) inductive tissues will be used as potential inducers as well as chemical agents which are known to affect other in vitro systems in which morphogenetic differentiation occurs (cAMP, IUdR, DMSO). The goal of this program is initially to determine whether the lesser tendency of fetal rat kidney (in comparison with that of the mouse) to form differentiated epithelial tumors after exposure to transplacental carcinogens is due to interspecies differences in cellular responsiveness to mediators of morphogenetic differentiation.

A major characteristic of the response of rodents to chemical carcinogens during intrauterine development is that susceptibility to neoplastic transformation generally is not demonstrable prior to completion of definitive organogenesis, which marks the beginning of the fetal period of development. True embryos, in which undifferentiated tissues are only beginning to form identifiable organ rudiments, are subject to teratogenic damage but are not in general at risk for subsequent tumor development as a consequence of exposure to carcinogens during this stage of development. The question arises whether this indicates (a) that neoplastic transformation fails to occur in embryonic tissues, or (b) that potential neoplastic transformants are generated, but are prevented from expressing their neoplastic genotype phenotypically by proliferation to generate a tumor. The latter might be accomplished through cell-cell interactions or other mediators of programmed normal differentiation to which cells altered by carcinogens are still responsive. A future project is planned using the fetal hamster, in cell cultures from which morphologic transformation of mesenchymal cells is demonstrable. Prenatal hamsters will be subjected transplacentally to a carcinogenic dose of a metabolism-independent carcinogen at different stages of development, from implantation of the blasto cyst through late fetal life. Cultures prepared from the conceptuses thus exposed will be studied for the presence of transformed cells, in an effort to demonstrate the presence of latent transformed cells in fetal tissues that appear refractory to carcinogenesis during early development. The fact that transformation is readily demonstrated in fetal hamster fibroblasts that originate from the soft connective tissues, in which tumors are not seen following transplacental exposure to carcinogens, strongly suggests that such an approach will be fruitful.

Publications

Rice, J. M.: Problems and perspectives in perinatal carcinogenesis. Natl. Cancer Inst. Monogr. 51: 271-278, 1979.

Perantoni, A. and Berman, J. J.: Properties of Wilms' tumor line (TuWi) and pig kidney line (LLC-PK_1) typical of normal kidney tubular epithelium. In Vitro 15: 446-454, 1979.

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Edwards, W. D., Berman, J. J., Rice, J. M., and Townsend, M. W.: Differentiation of a primary chemically induced rat nephroblastoma in organ culture. Devel. Growth Diff. 21: 518-525, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05094-02 LEP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

In vitro β-galactosidase synthesis from lac operon DNA: a model system of bacterial origin for study of DNA damage and repair by chemical carcinogens.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Pathology

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Perinatal Carcinogenesis Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.6	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

DNA, isolated from temperate coliphage λh80dlacp^S and containing the sequence coding for β-galactosidase, was used to initiate in vitro synthesis of the enzyme in the presence and absence of various carcinogens, especially alkylating agents. β-Galactosidase activity was measured colorimetrically by hydrolysis of O-nitrophenyl-β-D-galactopyranoside. Inhibition of synthesis of enzyme activity resulting from carcinogen interaction with DNA and with other components of the system is measured. Inhibition of β-galactosidase synthesis by methyl- and ethylnitrosourea is strictly a consequence of alkylation, and is equivalent at equal levels of total alkylation. This is inconsistent with hypotheses postulating that certain types of alkylation reaction such as 7-alkylguanine formation are insignificant. Carbamylation was not an important reaction with these compounds and, although demonstrable, did not contribute to inhibition of DNA function by methylnitrosourea.

PHS-6040
(Rev. 10-76)

Objectives: To study the consequences of carcinogen interactions with DNA in a system which yields a precisely defined gene product, in order to ascertain which carcinogen-DNA interactions result in inhibition of DNA function. To investigate the roles of various enzymes postulated to participate in excision repair of DNA by evaluating their capacity to restore function to lac DNA pretreated with various carcinogens and mutagens.

Methods employed: Temperate bacteriophage λ h80dlacp^s is thermally induced in E. coli host bacteria, purified by banding in CsCl and its DNA extracted. 30,000g Supernatants from homogenates of lac-deleted E. coli, plus salts and co-factors as described (Chem.-Biol. Interactions 14: 101-111, 1976), were used for synthesis of β-galactosidase using phage DNA as primer, with and without preincubation with alkylating agents or other carcinogens or mutagens. Enzyme synthesis is determined colorimetrically by hydrolysis of O-nitrophenyl-β-D-galactopyranoside. Alkyl-H^3 and alkyl-C^{14} methyl- and ethylnitrosoureas were purchased and used without further purification. Carbonyl-^{14}C methylnitrosourea was synthesized from KNCO-C^{14} and methylamine.

Major findings: In previous studies with this system, the carcinogenic and mutagenic alkylnitrosoureas MNU and ENU were shown to inhibit the ability of DNA to function as template for the synthesis of messenger RNA directing the synthesis of β-galactosidase when allowed to react directly with the DNA in the absence of competing substrates. Inhibition was shown to be directly proportional to the extent of total DNA alkylation, a result that contradicts the conventional view that certain sites of reaction in DNA, notably O^6-alklyl-guanine formation, are more important than others for the induction of lesions in DNA that are of genetic and biological significance. MNU and ENU have very different spectra of reaction products, and thus the fact that at equal levels of total alkylation they are equally effective inhibitors of β-galactosidase synthesis is not consistent with the proposition that a single alkylation product or a relatively few such products are the only reaction products of significance for genotoxicity.

Both MNU and ENU are carbamoylating agents as well as alkylating agents, by virtue of the fact that decomposition of either agent in aqueous solution yields the isocyanate anion, NCO^-. The significance of carbamoylation as an inhibitor of DNA template function and of the protein synthetic process was studied in this system. KNCO, when added to DNA at concentrations equimolar to that employed with MNU and ENU, had no effect on DNA template function. When added to the complete synthetic system, KNCO was significantly inhibitory, suggesting that carbamoylation of intracellular constituents other than DNA is a significant factor in the toxicity of these compounds, but does not contribute significantly to their genetic effects.

A major effort to study the function of endonucleases possibly active in DNA repair in this system was undertaken. The goal of this study was to correlate physical repair of damaged DNA with functional repair in capacity to direct synthesis of active enzyme. DNA was rendered partially apurinic by incubation at pH 4.0 at 37° for 3 hours followed by neutralization with 0.01 M Tris HCl

buffer (pH 8.0). Subsequent analysis of the apurinic DNA by alkaline sucrose gradient procedures confirmed that template activity for synthesis of -galactosidase was correlated with physical size of the DNA molecules. Apurinic DNA was attacked by nucleases present in the extract from E. coli employed in the protein synthesis system. Apurinic DNA was much more sensitive to degradation than native DNA, indicating the presence of apurinic endonucleases in this system. Inclusion of the 4 deoxyribonucleoside triphosphates in the reaction mixture significantly inhibited endonuclease activity, especially toward apurinic DNA. Work is now in progress to determine whether any of these deoxyribonucleoside triphosphates is incorporated into the apurinic DNA, which would consititute evidence for repair synthesis. An alternative explanation is that these substances are able nonspecifically to inhibibit nuclease activity, possibly as competing substrates for the enzyme, in which case the phenomenon would be of less interest.

Similar studies carried out with S-30 preparations from rodent tissues have indicated that much higher levels of apurinic endonucleases are present in fetal rat liver, an organ relatively resistant to transplacental carcinogenesis by alkylating agents, in comparison with very low levels that are present in fetal rat brain, an organ which is highly susceptible to transplacental carcinogenesis. This may possibly reflect a lower capacity for nucleic repair in the developing rat brain, which might contribute to its hightened susceptibility to carcinogens. However, studies with the mammalian enzyme systems are complicated by the fact that high levels of nucleases active on native DNA are also present, and identification of strictly apurinic endonuclease activity is much more difficult.

Significance to Biomedical Research and the Program of the Institute: The phenomenon of DNA repair, and its deficiency in genetic diseases such as xeroderma pigmentosum in which there is a high risk of cancer related to solar UV irradiation, provides the strongest evidence that direct damage to the cellular genome is one of the primary events in neoplastic transformation. The nature of DNA-carcinogen interactions, and the ability of cells to repair resulting damage, have been studied principally by physical methods which cannot demonstrate whether restoration of structural integrity to damaged DNA also results in restoration of normal function, which must generally be inferred indirectly. The existence of an in vitro system in which a well-defined functional gene product is synthesized from a specific DNA template may provide a means for directly determining which of the many DNA-carcinogen adducts in fact inhibit function. Furthermore, with the identification of precisely defined enzyme activities necessary for DNA repair in vivo, in vitro conditions may be established for assaying the roles of different candidate endonucleases in repair of damage by different classes of carcinogens, as well as differentiating between lesions that are accurately repairable and those which are not.

Proposed course: The potential value of this system for studies relevant to chemical carcinogenesis depends on the demonstration of not only DNA damage,

but DNA repair in this system. This has proved extremely difficult, and impossible to pursue effectively in this laboratory with the facilities and staff available. In accordance with the direction of the Laboratory, this project is being terminated this fiscal year. This will be the final report on the use of this system in this Section.

Publications

Wei, S. C., Chen, B. P., and Rice, J. M.: Comparative effects of methyl- and ethylnitrosourea on DNA directing cell-free DNA-dependent synthesis of β-galactosidase. Mol. Pharmacol., in press (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05106-02 LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Human Tissue and Cell Mediated Mutagenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I. Hsu	Expert	LEP,NCI
Other:	H. Autrup	Senior Staff Fellow	LEP,NCI
	C. Harris	Assoc. Chief, LEP and Chief, HTSS	LEP,NCI

COOPERATING UNITS (if any)
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University of Maryland, School of Medicine, Baltimore, MD

LAB/BRANCH
Laboratory of Experimental Pathology (LEP)

SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human bronchial explants, pulmonary alveolar macrophages (PAM) and blood monocytes metabolically activated benzo[a]pyrene (B[a]P) and 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (7,8-diol) to ultimate mutagens. Using a human tissue- and cell-mediated mammalian mutagenesis assay these activated metabolites caused increases of ouabain resistant (0^r) mutation frequency and number of sister chromatid exchanges (SCE) in the cocultivated indicator cells (Chinese hamster V-79 cells). Ouabain resistant mutation, SCE, nascent DNA synthesis and cytotoxicity were compared in V-79 cells treated with dihydrodiol epoxide derivatives of B[a]P. We found that inhibition of nascent DNA synthesis by these direct carcinogens was more closely related to cytotoxicity than to the induction of SCE or 0^r mutations. Caffeine inhibited nascent DNA synthesis and enhanced cell killing but did not influence the resulting induced levels of mutagenesis or SCE. Analysis of our data concerning the induction of SCE indicates that enhancement of SCE frequency by an agent, e.g., H_2O_2 and trans-PT(II) does not necessarily lead to increase in mutation frequency.

Objective: To study mutation of V-79 cells caused by chemicals and mediated by human tissues or cells. The relationship, if any, between mutagenicity mediated by human cells and oncogenic susceptibility among individuals will be assessed. To determine the biological significance of sister chromatid exchanges, their mechanism of formation and their relationship to DNA damage and mutagenesis in Chinese hamster V-79 cells.

Methods Employed: Explant cultures of human tissues; preparation of PAM from lung tissue and bronchial lavage; isolation and culture of human and blood monocytes; cocultivation of V-79 cells (a Chinese hamster cell line) and human tissues in medium containing a suspected chemical carcinogen; selection of V-79 cell mutant by media containing either ouabain or 8-azaguanine; Hoechst-Giemsa chromosome stain to determine sister chromatid exchanges; isolation of carcinogen-DNA adducts in human tissues. Treatment of V-79 cells with several different types of direct-acting chemical and physical mutagens; selection of the mutants by medium containing ouabain, 8-azaguanine or 6-thioguanine; alkaline sucrose gradient centrifugation for the determination of DNA postreplication repair; alkaline elution for measurement of DNA damage in the cells.

Major Findings: Cultured human bronchial explants activated B[a]P into electrophilic metabolites that bind to DNA in bronchial epithelial cells. Promutagenic and mutagenic metabolites of B[a]P were also released into the culture medium. An increase in mutation frequency for O^r was found in Chinese hamster V-79 cells when they were co-cultivated with bronchial explants in the presence of B[a]P. The proximate carcinogenic form of B[a]P, 7,8-diol ((+)*r*7, *t*8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene), was 5-fold more potent as a promutagen than the parent compound. Neither B[a]P nor 7,8-diol increased the O^r mutation frequency in V-79 cells when they were cultured without bronchial explants. The mutation frequency was directly related to the binding levels of B[a]P to bronchial DNA and the concentration of either B[a]P and 7,8-diol, and their metabolites were released into the culture medium. Increases in the frequency of ouabain-resistant (O^r) mutations and sister chromatid exchanges were found in V-79 cells only when they were cocultivated with both PAM and chemical procarcinogens. 7,8-Diol caused higher frequencies of both O^r mutations and sister chromatid exchanges than did the parent compound, B[a]P. When metabolically activated by PAM the mean O^r mutation frequency caused by B[a]P was nine O^r mutants per 10^6 surviving V-79 cells per 10^6 PAM and a 10-fold interindividual variation (range 2-21) was found. The mean O^r mutation frequency caused by 7,8-diol was 64 and a 9-fold interindividual variation (range 14-120) was found. In the absence of PAM, the O^r mutation frequency in V-79 cells was 0.5 ± 0.2 O^r mutant per 10^6 survivors. 7,8-Benzoflavone, an inhibitor of mixed function oxidases, reduced the frequencies of O^r mutations and of sister chromatid exchanges in V-79 cells caused by 7,8-diol and by B[a]P. As expected, 7,8-benzoflavone did not influence the frequency of O^r mutations caused by one of the ultimate mutagens derived from B[a]P and 7,8-diol, (+)7R,8S-dihydroxy-9R,10R-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Blood monocytes in culture can mediate mutation in V-79 cells. Although monocytes did not have activity as high as PAM to mediate mutation in V-79 cells, monocyte mediated O^r mutation was dependent on the number of monocytes used and the concentration of 7,8-diol.

Human pulmonary macrophages were able to phagocytize B[a]P adsorbed on ferric oxide particulates in a time-dependent manner. B[a]P was eluted from the engulfed particulates and released into the extracellular space as either free B[a]P or as metabolites of B[a]P. Some of the released metabolites were mutagenic for cocultivated Chinese hamster V-79 cells. A higher binding level of B[a]P to bronchial DNA was found when bronchial explants were co-cultivated with macrophages containing B[a]P-ferric oxide than when incubated with B[a]P-ferric oxide alone.

The analysis of SCE is often proposed as a sensitive and quantitative assay for genetic damage of the sort leading to mutation and/or cancer. We have found agents that produce varying types of DNA damage but an undetectable increase in mutation frequency. We wondered whether any of these agents would produce SCEs, and if so, whether any correlation could be drawn between DNA damage and SCE formation. In V-79 cells, mutation was measured as resistance to 6-thioguanine, toxicity was measured by the loss of plating efficiency, and DNA damage was measured by alkaline elution. The results show that the undetectably mutagenic agents, transPt. (II) diammine-dichloride, visible light from a GE 275w sunlamp above 350 nm (1.8×10^5 J m^{-2}), and hydrogen peroxide produce significant increases in SCE frequency (2.911 ± 0.131, 1.556 ± 0.139, 0.616 ± 0.048 per chromosome, respectively; control, 0.351 ± 0.230). The mutagenic agents, cis-Pt. (II) diammine-dichloride and the sunlamp at wavelengths above 305 nm (2.4×10^5 J m^{-2}), also produced large increases in SCE frequency (3.960 ± 0.200 and 1.722 ± 0.135 exchanges per chromosome). DNA-DNA crosslinks were produced only by the Pt. complexes, while DNA single strand breaks were produced by the other agents. DNA-protein crosslinks were produced by all agents except H_2O_2. Thus, so far no consistent correlation between various DNA lesions and SCEs has been found. We conclude that lesions leading to SCEs and certain types of DNA damage will not necessarily produce mutations.

To study the relationship of mutation, SCE, cytotoxicity and nascent DNA synthesis, V-79 cells were treated with B[a]P diol epoxide I and II. Both isomers caused dose-dependent decreases in survival of cells and in the size of nascent DNA. Increases in the frequencies of SCE and of 0^r mutations were found in cells treated with either isomer. However, B[a]P diol epoxide I caused 10-20-fold more 0^r mutations and 100% more SCE than did B[a]P diol epoxide II at equal molar dose levels. In contrast, the isomers induced similar reductions in the size of the nascent DNA at equal dose levels. Caffeine, at non-toxic dose levels, caused a synergistic reduction in cell survival induced by both isomers and also inhibited DNA elongation. However, caffeine did not affect the induction of either 0^r mutants or SCE by either isomer. Thus the results suggest that the reduction in the size of nascent DNA by both isomers was more closely related to cytotoxicity than to the induction of SCE or 0^r mutations.

Significance to Biochemical Research and the Program of the Institute:
Most studies with chemical carcinogens were done in experimental animals. Extrapolation of the results to humans is a complex problem, in part because of the differences in the metabolism of chemicals between species. The mutagenesis test described above will utilize human tissue from several

different organs and individuals, and may provide clues in the search for host factors that influence oncogenic susceptibility. Several methods are used for screening chemical carcinogens. To evaluate the validity of the tests or the significance of the results, it will be helpful to understand the mechanism of the test and its relation to carcinogenesis. These studies of DNA damage, DNA repair synthesis, and sister chromatid exchange will provide information on the mechanisms of sister chromatid formation.

Proposed Course of the Project: Continuation of the mutation studies mediated by human tissues and cells. Use of this system to test carcinogens other than polynuclear aromatic hydrocarbons and expand the test to any environmental pollutants. Measurement and comparison of SCE, DNA repair synthesis, and mutation of V-79 cells mediated by human tissue. Development of the human tissue and cell mediated transformation and mutation assay with human cells as the detector system.

Publications

Autrup, H., Harris, C. C., Trump, B. F., Stoner, G. D., and Hsu, I. C.: Uptake of benzo[a]pyrene-Fe_2O_3 particulates by human pulmonary macrophages and release of benzo[a]pyrene and its metabolites. Proc. Soc. Expt. Biol. Med. 161: 280-284, 1979.

Hsu, I. C., Harris, C. C., Trump, B. F., Schafer, P. W., and Yamaguchi, M.: Induction of ouabain-resistant mutation and sister chromatid exchanges in Chinese hamster cells with chemical carcinogens mediated by human pulmonary macrophages. J. Clinical Invest. 64: 1245-1252, 1979.

Hsu, I. C., Bowden, G. T., and Harris, C. C.: A comparison of cytotoxicity, ouabain-resistant mutation, sister chromatid exchanges and nascent DNA synthesis in Chinese hamster cells treated with dihydrodiol epoxide derivatives of benzo[a]pyrene. Mutation Res. 63: 351-359, 1979.

Bradley, M. O., Hsu, I. C., and Harris, C. C.: Sister chromatid exchanges: Varying relationship to mutagenicity, toxicity and DNA damage. Nature 282: 318-320, 1979.

Bowden, G.T., Hsu, I.C., and Harris, C.C.: The effect of caffeine on cytotoxicity, mutagenesis and sister chromatid exchanges in Chinese hamster V-79 cells treated with dihydrodiol epoxide derivatives of benzo[a]pyrene. Mutation Res. 63: 361-370, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05130-01 LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Carcinogenesis Studies on Human Epithelial Cell Cultures

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J. Lechner	Expert	LEP,NCI
	Å. Haugen	Guest Worker	LEP,NCI
	C. Harris	Assoc. Chief, LEP and Chief, HTSS	LEP,NCI
Other:	H. Autrup	Senior Staff Fellow	LEP,NCI

COOPERATING UNITS (if any)

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Litton Bionetics, Inc., Kensington, MD

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methods to grow replicative cultures of normal human bronchial epithelial cells have been developed. These cells, which can be subcultured and will undergo 35 population doublings, have the expected epithelial cell characteristics of keratin, desmosomes, blood group antigens and the normal chromosomal karyotype. They form colonies when plated at low cell density. Two percent serum and mouse 3T3 feeder cells are required for growth; addition of EGF, fibronectin and cholera toxin markedly increase the clonal growth rate and cloning efficiency. Explant cultures of human bronchus have been maintained for more than one year. In addition, long-term continuous growth of epithelial cells has been accomplished by repeated transfer of a tissue explant. After 6 months and 13 transfers, epithelial cells continue to grow out from both control and carcinogen treated explants. Human prostatic epithelial cells previously infected with SV40 virus have been repeatedly exposed to 4NQO. The carcinogen treatment caused extensive aneuploidy. The tumorigenic potential of these cells is being assessed in athymic nude mice.

Objectives: To develop systems to study malignant transformation of human epithelial cells. These studies include the following: (1) development of replicative cell cultures from bronchial, esophageal, prostatic and colonic tissue; (2) development of improved media for growth of human epithelial cell cultures; (3) evaluating both long-term explant and rapidly dividing cell cultures as model systems to study in vitro malignant transformation; (4) evaluating carcinogenic exposure of xenotransplanted subsegmental bronchi as a model system to study human carcinogenesis; and (5) studying the interactive effects of chemical and physical carcinogens with co-carcinogens and virus in vitro.

General Methods: Human bronchial, esophageal and colonic tissues are obtained from surgery and "immediate" autopsy. Normal and SV40 infected human prostatic epithelial cultures were developed previously by the P.I. while at the Pasadena Foundation for Medical Research. Bronchial and esophageal tissues are dissected from surrounding stromal tissues, cut into 0.5 cm square pieces which are then used to establish explant cultures. Colon epithelial tissue, freed from its underlying stroma, is minced, then disaggregated by digestion with various enzymes. The cells are then placed on supports, e.g., gel foam, polycarbonate filters, collagen rafts, etc., and incubated in various media.

Two types of long-term explant cultures are being used to scrutinize cellular changes induced by exposure to chemical carcinogens. Quasiquiescent long-term explant cultures maintained for more than 1 year and exposed to 4-nitroquiniline-1-oxide (4NQO) both with and without asbestos are examined periodically for foci of mitotic epithelial cells. Further, to elucidate the interaction between epithelial cells and asbestos, cells have been grown onto grids and then exposed to asbestos for 3 weeks. These cells are being studied using high-voltage and scanning electron microscopy techniques. Explant cultures are also used to study carcinogenesis in a continually growing epithelium. After epithelial cell migration, the explant is transferred to a new dish. Both the outgrowth and transplanted explant dishes are maintained. After a new outgrowth, the explant is again transferred. This protocol is repeated indefinitely. The explants are repeatedly exposed to either 7,12-dimethylbenz[a]anthracene, DMBA (weekly) or 4NQO (biweekly).

Normal human prostate epithelial cells, experimentally infected with SV40 virus, exhibited several abnormal (transformed) cellular characteristics. Subsequently, these cells were exposed for six weeks to a half lethal dose of 4NQO, then xenotransplanted, both subcutaneously and into the immunologically privileged anterior chamber of the eye. Similar nickel-SV40 virus co-carcinogenesis experiments are also in progress. In addition, experimental viral/ chemical co-carcinogenesis studies of bronchial epithelium has been initiated.

Several criteria are being used to establish the normal and epitheloid natures of the cells grown in culture. Establishing markers to identify normal epithelial cells in vitro has been an important objective for this laboratory. These efforts have identified several criteria,

among which are normal karyology, polygonal morphology, ciliary activity, scanning electron microscopic morphology, ultrastructural identification of tight junctions, desmosomes and tonofilaments, production of acidic and neutral mucopolysaccharides, and immuno-staining of keratin, blood group antigens and type IV collagen, population doubling potential, clonal growth rate and mitogenic responsiveness to peptide growth factors and hormones. Changes in these cellular properties as well as abnormal exfoliative cytology and anchorage independence, are being sought as a result of exposure to chemical carcinogens. Ultimately, growth in athymic nude mice will be tested.

As a model system for in vivo carcinogenesis, small sub-segmental bronchi are implanted subcutaneously into nude, athymic mice. After vascularization, carcinogens incorporated into beeswax pellets or absorbed on lycra fibers are inserted into the lumen. Periodically these tissues are recovered and assessed by pathological examination.

Major Findings: Human bronchial epithelial cell culture experiments have yielded: (1) A method for routinely initiating replicative epithelial cell cultures of human bronchus has been developed. Large pieces of bronchus tissue are initially set-up as explant cultures and incubated in a rocking chamber for two to three days to facilitate removal of mucus. These explants are then cut into smaller pieces and explanted into medium containing less than 2% serum. The low serum concentration permits rapid outgrowth of epithelium but retards growth of the fibroblastic cells. Thus, after 5-10 days incubation, very few fibroblastic cells are present among the epithelial outgrowth. At this time the tissue explant is transferred to a new dish for reseeding a second wave of epithelial cell outgrowth. The epithelial cells remaining on the dish cease dividing and eventually die unless Swiss mouse 3T3 feeder cells are added to the cultures. These feeder cells-supported bronchial epithelial cells continue to divide rapidly. These cells have the normal human karyotype; (2) The human bronchial epithelial cells have been subcultured at low density and clonal growth has been accomplished. Feeder cells and 2% serum supplement were necessary for growth of the subcultured cells; (3) Coating the dish surface with fibronectin and adding epidermal growth factor (EGF) and cholera toxin to the nutrient medium improves clonal plating efficiency (from 2 to 15%) and increases the clonal growth rate (2.5-fold). The addition of other factors, e.g., MSA, FGF and EGGS were less mitagenic; (4) Cultures of human bronchial epithelium average 35 population doublings in vitro; (5) Epithelial cell markers have been demonstrated. Keratin was shown by both immunospecific, Kreyberg and rhodamine B staining. Blood group antigens were also detected by immunospecific staining. However, mucopolysaccharide staining was negative. Ultrastructurally, these cells have numerous tonofilaments and desmosomes; (6) The calcium concentration of the medium influences colony morphology and cell shape. The cells are small and tightly polygonal when growing in normal (1 mM) levels of calcium. Progressive reduction of the calcium concentration to 0.06 mM causes the cells to become flatter and less closely associated; (7) EGF decreases the number of desmosome junctions between cells. Examination of in situ fixed epithelial colonies revealed markedly less desmosomes, compared with colonies grown without EGF.

Growth experiments with esophageal epithelial cells have indicated: (1) Methods used to initiate cultures of bronchus have been successfully adapted for esophageal epithelial cultures. Up to three successive cultures have been initiated by transfer of the tissue fragment; (2) These cell cultures continue to proliferate only if supported by 3T3 feeder cells; and (3) These cultures have been successfully subcultured although the plating efficiency was less than 0.1%.

Preliminary growth experiments with colonic epithelium have indicated: (1) Explant cultures of colonic tissue routinely fail to develop outgrowths; (2) Enzymatically dissociated cells routinely fail to attach to plastic culture dish surfaces, although some attachment was observed on polycarbonate filters (5 μpore) and under a collagen gel. However, no growth was noted.

In vitro carcinogenesis results with long-term cultures of human bronchial tissue are: (1) Explant cultures have been maintained for more than 1 year. A steady state condition has been established whereby dying cells are replaced by cells migrating from the tissue. The older cultures consist predominantly of spindle-shaped cells; (2) Thirteen successive epithelial cell cultures have been initiated by repeatedly transferring a tissue fragment. After 6 months, epithelioid cells are still the prominent cell type migrating out from the tissue. However, fibroblastic cells become dominate in these cultures within two months after transfer of the tissue fragment unless feeder cells are added to the cultures. After eleven transfers, secondary cultures of carcinogen treated cells grow more rapidly with feeder cells than secondary cultures of nonexposed control cells. The exposed cells retained the normal karyotype through at least 9 transfers; (3) Asbestos fibers rapidly penetrate the plasma membrane of the epithelial cells. High voltage and scanning electron microscopy studies, still in progress, have shown asbestos in the cytoplasm, but many fibers were protruding through the plasma membrane after 24 hrs incubation; (4) Carcinogen treatment caused the modal chromosome number of SV40 infected human prostatic epithelial cells to increase from 46 to more than 60. These exposed cells have been xenotransplanted; (5) The in vivo carcinogenesis investigations are still in progress. Polonium 210 has been implanted into the lumen of several xenotransplanted subsegmented bronchi. Serial-sacrifice studies will begin within a few months.

Significance to Biomedical Research and to the Program of the Institute: The extrapolation of experimental animal data to man is a major problem in carcinogenesis. One approach to provide a link between these experimental data and human cancer is to develop model systems in cultured human tissues for carcinogenesis investigations. Such systems could be used for the identification of carcinogens and their metabolic pathways and, ultimately, in the identification of individuals who are highly susceptible to chemical carcinogens.

Proposed course of the Project: Growth conditions for human epithelial cells developed from bronchial, esophageal, and colonic tissues, will be continually improved. Characterization of the growth properties of these

cells is underway. Experiments are in progress to determine whether carcinogen treated bronchial epithelial cells lose antigens commonly associated with normal cells and demonstrate the growth characteristics ascribed to transformed cells. Changes in these properties may be indicative of pre-malignant transformation. Investigations on single and multiple carcinogen exposures as well as co-carcinogenesis with physical and viral agents on the development of neoplastic lesions in explant and cell cultures and xenotransplanted tissues will be continued. Ultimately, the mechanisms through which carcinogens transform epithelial cells will be investigated.

Publications

Stoner, G.D., Harris, C.C., and Myers, G.A.: Putrescine stimulates growth of human bronchial epithelial cells in primary culture. In Vitro, in press, 1980.

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Hillman, E.A., Vocci, M.J., Combs, J.W., Sanefugi, H., Robbins, T., Janss, D.L., Harris, C.C., and Trump, B.F.: Human Breast Organ Culture Studies. In Cultured Human Tissues and Cells, Volume II, in the series "Methods in Cell Biology," Volume 21B. New York, Academic Press, in press, 1980.

Harris, C.C.: Culture of Human Respiratory Tissues and Cells, an Overview. In Harris, C.C., Trump, B.F., and Stoner, G.D. (Eds.): Cultured Human Tissues and Cells, in the series "Methods in Cell Biology," Volume 21A. New York, Academic Press, in press, 1980.

Valerio, M.G., Fineman, E.L., Bowman, R.L., Harris, C.C., Trump, B.F., Hillman, E.A., and Heatfield, B.M.: Preliminary studies of normal untreated and/or carcinogen-treated adult human breast, prostate and esophagus as xenografts in nude mice. Proc. Third International Workshop, Gustav Fischer Verlag, in press, 1980.

Stoner, G.D.: Explant Culture of Human Peripheral Lung. In Harris, C.C., Trump, B.F., and Stoner, G.D. (Eds.): Cultured Human Tissues and Cells, in the series "Methods in Cell Biology," Volume 21A. New York, Academic Press, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE. PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-CP-05131-01 LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
DNA Damage by Chemical Carcinogens and its Repair in Human Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Fornace, Jr.	Expert	LEP,NCI
Other:	J. Lechner	Expert	LEP,NCI
	C. Harris	Assoc. Chief, LEP and Chief, HTSS	LEP,NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Experimental Pathology (LEP)

SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our initial studies have involved the effect of asbestos and several metal carcinogens, which are often present in trace levels in asbestos, on DNA in human cells. With the alkaline elution technique, DNA-protein crosslinks were detected in fibroblasts exposed to chromate salts. No evidence of repair was seen with up to 12 hours repair incubation after chromate removal from the culture medium. Using an in vitro assay, DNA-protein crosslinks were also detected. In the case of asbestos and nickel, no DNA damage was seen after exposure of fibroblasts to these agents. With the development of methods to maintain human bronchial epithelial cells in culture, DNA damage and repair studies are now being initiated with these cells with particular emphasis on agents implicated in pulmonary carcinogenesis.

Object: The purpose of our investigations is to study DNA damage induced by chemical and physical carcinogens and its repair in human cells in culture. The effect of certain chemical carcinogens on the repair of DNA damage induced by other carcinogens will also be determined.

Methods: Human fibroblasts were obtained from the American Type Tissue Culture Collection and maintained in culture for approximately 25 generations. Methods for the establishment of primary cultures of human bronchial epithelial cells are described in Project Number Z01-CP-05130-01-LEP. DNA single strand breaks and DNA crosslinks were assayed by alkaline elution. With this technique, as few as 200 DNA single strand breaks, DNA-protein crosslinks, or DNA interstrand crosslinks per cell can be detected. DNA-protein crosslinks, produced in vitro, are measured by a filter assay which selectively binds protein and DNA crosslinked with protein but not free DNA (Braun, A., Photochem. Photo Biol. 21: 243, 1975). Repair synthesis is measured by incorporation of labeled thymidine into cells treated with hydroxyurea and arginine deprivation.

Major Findings: This is a new project. Initial studies were directed at the effect of asbestos and several metal carcinogens on cellular DNA. Human fibroblasts were treated with these agents and DNA damage determined by alkaline elution. When these cells were incubated with asbestos (crocidolite) for up to 20 hours, no DNA strand breaks or other lesions were detected in the cells, even though the fibers appeared to have penetrated the cell membrane and were toxic to the cells. A similar result was also seen with nickel (N_i^{+2}). Studies are presently underway with cadmium salts.

In the case of chromate, $K_2C_rO_4$, treatment of human fibroblasts with this carcinogenic agent produced the interesting observation that high levels of DNA crosslinks had been produced. If prior to alkaline elution the cell lysates were digested with proteinase K, this effect was removed, indicating that these crosslinks are DNA-protein in nature. A similar effect has been seen with known DNA-protein crosslinking agents such as formaldehyde, trans-Pt (II) diammine-dichloride (trans-Pt), ultraviolet radiation, etc. The crosslinking effect induced by chromate was quite large--in the same range as that produced by formaldehyde or trans-Pt. No removal of these crosslinks was observed with up to 12 hours of incubation in chromate-free medium after doses of chromate from 25 μM for 2 hours to 200 μM for 2 hours. Previous reports indicate that the lower dose (25 μM) is not highly toxic to human fibroblasts with a colony survival of greater than 10-20%. A low level of DNA single strand breaks, approximately 1,500 per cell, was also seen after chromate treatment; unlike the crosslinks these breaks appeared to be rapidly "repaired" with complete removal by 4 hours. No effect was seen with chromic (Cr^{+3}) compounds or with chromate reduced to chromic with bisulfite. A similar level of crosslinks was also detected in human bronchial epithelial cells treated with chromate. Initial studies indicate that repair synthesis can be detected with chromate treatment as previously reported (Chem.-Biol. Interactions, 26: 267, 1979). DNA protein crosslinks were also detected in vitro with chromium salts.

Significance to Biomedical Research and the Program of the Institute: Chromate salts have been shown to be carcinogenic in laboratory animals and in human epidemiological studies. It will also transform cells in vitro to a malignant phenotype. I have recently shown that another DNA-protein crosslinking agent, trans-Pt, will also induce malignant transformation. The crosslinking effect induced by chromate may play a role in its carcinogenic activity. Studies have recently been initiated using human bronchial epithelial cells. The effect of asbestos and other agents, which have been implicated in pulmonary carcinogenesis, can now be studied in culture with the clinically relevant cell type.

Proposed course of the project: Studies will be continued with chromate in an attempt to characterize the DNA-protein crosslinks generated in vitro After chromate damage, repair synthesis will be determined in excision repair proficient and deficient cells using methods previously described and BND cellulose chromatography. Further studies will be undertaken to determine if chromate-induced crosslinks are repaired in normal cells. With the development of cell culture of human bronchial epithelial cells, several studies can now be undertaken using the cell type which is the apparent origin for a very common human malignancy. DNA damage and repair will be monitored in these cells using the methods previously outlined. The direct effect of asbestos on these cells can be determined. From epidemiologic studies, asbestos appears to potentiate the carcinogenic effect of cigarette smoking; the effect of asbestos on DNA damage by polycyclic aromatic hydrocarbons will be studied with emphasis on the effect of asbestos on carcinogen uptake, DNA damage, and DNA repair. The incision, repair synthesis, and ligation steps of DNA excision repair will be monitored and the possible effect of agents such as asbestos on these processes will be determined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05132-01 LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Ultrasensitive Enzymatic Radioimmunoassay

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I. Hsu	Expert	LEP,NCI
	C. Harris	Assoc. Chief, LEP and Chief, HTSS	LEP,NCI
Other:	G. Trivers	Research Scientist	DCCP,NCI
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Institute for Cancer Research, Columbia Ponds, New York, NY

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SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

By combining radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), we have developed a very sensitive immunoassay -- ultrasensitive enzymatic radioimmunoassay (USERIA). Experiments were conducted to modify RIA or ELISA so that antigens or antibodies could be measured by the USERIA procedure, and to compare the sensitivity among these immunoassays. So far USERIA has proven to be the most sensitive immunoassay. It is 100- to 1,000-fold more sensitive than RIA in the detection of rotavirus, cytomegalovirus, and cholera toxin. The procedure was modified to determine covalent binding of chemical carcinogens to DNA. The USERIA procedure which measures benzo[a]pyrene (B[a]P)-DNA adducts and acetylaminofluorene (AAF) adducts was 100-fold more sensitive than RIA. As low as 3 fmole AAF DNA adducts in 10 ng DNA can be measured by non-competitive USERIA and 2 fmole in 1 µg DNA by competitive procedures. With a similar procedure, 4 fmole of B[a]P-DNA adducts in 10 ng DNA can be detected by non-competitive USERIA, and 10 fmole in 25 µg DNA by competitive methods.

Objective: A sensitive method that can directly measure DNA damage or covalent binding of carcinogens to cellular macromolecules is needed for both clinical and molecular studies. The USERIA procedure has been worked out to detect fmole levels of B[a]P-DNA adducts and AAF DNA adducts. We will use the method to measure the binding of carcinogens found in the polluted environment to DNA in human cells or tissues, e.g., pulmonary macrophages or lung tissues.

Methods Employed: DEAE Sephadex column chromatography for separation of ^{3}H-AMP; enzyme linked immunosorbent assay (ELISA); radioimmunoassay (RIA); tissue or cell cultures; DNA-isolation from cultured bronchial explants; preparation of alkaline phosphatase IgG conjugates.

Major Finding: USERIA combines the advantages of ELISA and RIA; it has the simplicity of ELISA and is more sensitive than RIA. The use of ^{3}H-adenosine-5'-monophosphate (AMP) as substrate increased detectability of alkaline phosphatase 10,000-fold over the use of ρ-nitrophenylphosphate. By modifying the ELISA procedure and using ^{3}H-AMP as substrate, one can improve the signal to noise ratio and achieve a much higher sensitivity. USERIA can used in several areas. Clinically it has been proved to be the most sensitive immunoassay and can detect several infectious viruses in specimens from infected patients, e.g., CMV, which could not be detected by RIA and ELISA. In chemical carcinogenesis studies, USERIA can be used to determine the damage of carcinogens to DNA, i.e., covalent binding of AAF or B[a]P to DNA. As few as 10 fmole of B[a]P-DNA adducts can be measured in 25 μg DNA by competitive USERIA and 4 fmole in 10 ng DNA by non-competitive procedure. For AAF DNA adducts, USERIA can detect approximately 2-3 fmole in 1 μg DNA by competitive method and 3 fmole in 10 ng DNA by non-competitive procedures. The assay should be sensitive enough to measure the B[a]P binding to DNA of human cells or tissue exposed to carcinogens. In addition, it is a simple technique to detect antibody production by monoclonal hybridomas and to measure the antibody titer.

Significance: The radiochemical method has been a convenient technique for studying the metabolism of chemical carcinogens and their subsequent binding to DNA in experimental animals and in cultured cells. However, chemical carcinogens in the polluted environment are not generally radioactive. To study the DNA damage by these contaminated chemicals, assays other than radioisotope techniques must be used. During the last few years, antisera against benzo[a]pyrene modified DNA and acetylaminofluorene modified DNA have become available. RIA appears to be the most sensitive assay, other than the radioisotope technique, with which to measure the carcinogen-DNA adducts. However, to detect covalent binding of B[a]P to DNA in biological samples from people exposed to polluted carcinogens, improvement of the sensitivity of RIA is required. We have now modified RIA and increased the sensitivity 100-fold. USERIA is sensitive enough to measure the B[a]P-DNA adducts in human tissues or cells.

Proposed Course of the Project: To determine if B[a]P-DNA adducts can be detected by USERIA in pulmonary alveolar macrophages or biological

samples from asphalt workers, coke oven workers and persons exposed to cigarette smoke.

Publications

Harris, C.C., Yolken, R., Krokan, H., and Hsu, I.-C.: Ultrasensitive enzymatic radioimmune assay (USERIA): Application for quantitative detection of cholera toxin and rotavirus. Proc. Natl. Acad. Sci. 76: 5336-5339, 1979.

Hsu, I.C., Poirier, M., Yuspa, S., Yolken, R., and Harris, C.C.: Ultrasensitive enzymatic radioimmunoassay can detect femtomoles of acetylaminofluorene-DNA adducts. Carcingenesis, in press, 1980.

Hsu, I.C., Yolken, R., and Harris, C.C.: Ultrasensitive Enzymatic Radioimmunoassay. In Methods in Enzymology. New York, Academic Press, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05133-01 LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Monoclonal Antibodies to Carcinogen-DNA Adducts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Haugen	Guest Worker	LEP,NCI
	I. Hsu	Expert	LEP,NCI
	C. Harris	Assoc. Chief, LEP and Chief, HTSS	LEP,NCI
Others:	J. Lechner	Expert	LEP,NCI
	H. Autrup	Senior Staff Fellow	LEP,NCI

COOPERATING UNITS (if any)
Massachusetts Institute of Technology, Cambridge, MA
Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland

LAB/BRANCH
Laboratory of Experimental Pathology (LEP)

SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse myeloma cells (P_3x63) were fused with spleen cells from Balb/c mice immunized with AFB_1-DNA adducts. Hybrid cells were grown in selective medium and tested for production of antibody-secreting hybridomas. Clones secreting monoclonal antibodies binding specifically to AFB_1-DNA adducts have been obtained.

Objectives: Monoclonal antibodies will be used to detect carcinogen-DNA adducts in human tissue specimens and cells and will be used in experiments such as 1) determination of exposure of individuals to chemical agents; 2) adduct-distribution in different organs; 3) DNA repair studies; and 4) experimental *in vitro* carcinogenesis.

Methods: Spleens from immunized mice are removed 2-3 days after the last immunization and minced in Dulbecco's phosphate buffered saline. Spleen cells (10^8) are mixed with myeloma cells (10^7), fused with polyethyleneglycol and grown in selective medium. Myeloma cells will not grow in the selective hypoxanthine/aminopterin/thymidine (HAT) medium. Since spleen cells will not grow in culture, the only cells that survive are cell hybrids. Hybrid cells are dispersed in 96 well plates and incubated at 37°C. Cell growth after 14 days is taken as a successful hybrid. Cells can then be cloned with thymus cells added to the well as feeder cells. Supernatant from these clones is assayed with a modified enzyme-linked immunosorbent assay. Cells producing specific antibody are recloned and then expanded and injected i.p. into mice for development of ascites tumor. Monoclonal antibodies from ascites and cell culture medium are then isolated and characterized.

Results: This is a new project. Immune response to AFB_1-DNA adducts were obtained by injecting into mice methylated bovine serum albumin-AFB_1-DNA conjugate emulsified in Freund's complete adjuvant. Clones producing monoclonal antibodies against AFB_1-DNA adducts have been obtained.

Significance to Biomedical Research and to the Program of the Institute: Methods are being developed to quantitatively measure carcinogen-DNA adducts in femtomole and less amounts. These methods will be useful in studies of the molecular interactions of carcinogens and cell genome and in measurement of carcinogen-DNA adducts in biopsy specimens from people in high and low risk environments for cancer.

Proposed course: Studies are under way to further characterize this antibody and also to make monoclonal antibodies which specifically recognize other carcinogen-DNA products. These monoclonal antibodies and ultrasensitive enzymatic radioimmunoassay will be utilized to search for carcinogen-DNA adducts in human biopsy specimens and to determine the intra- and intergenomic distribution of these adducts.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05134-01 LEP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Enzyme Immunoassay and Biological Function of Epidermal Growth Factor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Trivers	Research Scientist	DCCP,NCI
Others:	C. Harris	Assoc. Chief, LEP and Chief, HTSS	LEP,NCI
	I. Hsu	Expert	LEP,NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Experimental Pathology (LEP)

SECTION
Human Tissue Studies Section (HTSS)

INSTITUTE AND LOCATION
NCI,NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.6	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse Epidermal Growth Factor (mEGF) stimulates cellular proliferation and differentiation in infant mice and in a wide variety of cell cultures. Establishing the sites of synthesis and the conditions effecting the secretion of this polypeptide will require assays with extreme sensitivity. The recent development in this laboratory of the ultrasensitive enzymatic radioimmunoassay (USERIA), significantly more sensitive than RIA, provides the potential of the necessary sensitivity. A modification of the enzyme-linked immunoabsorbent assay (ELISA) has been employed to determine the preliminary conditions for applying the USERIA to the study of mEGF. Mouse EGF is coated onto the wells of microtiter plates and reacted with specific antibody. Heterologous antibody conjugated to alkaline phosphatase is then added. Subsequently, a colorimetric substrate (ELISA) or tritiated adenosine-5'-monophosphate (USERIA) is added as substrate to quantitate the results. A variety of experimental designs have been used to determine the major requirements for optimum conditions in a competitive USERIA.

PHS-6040
(Rev. 10-76)

Objectives: To adapt the USERIA for the study of mEGF and hEGF in normal tissues and during the induction of chemically-induced malignant cell transformation.

Methods: Commercially produced, highly purified mEGF and specific, polyclonal rabbit anti-EGF antisera; modified enzyme-linked immunoabsorbent assay (ELISA) for optimization of coating conditions (diluents, temperature, incubation time, etc.), and reagent concentrations and reaction times; comparative study of ELISA and USERIA to determine the ultimate conditions for maximum sensitivity in the competitive USERIA.

Major Findings: This is a new project. Preliminary results established the mEGF plate coating time from 16-24 hours at 37°C; beyond this period the immunoreactivity rapidly decreases with time. Increases in the hydrolysates have been shown proportional to increases in the amounts of mEGF in both assays. When 100 pg of EGF are coated on the plate, the addition of competitive mEGF to the antibody solution causes inhibition of enzyme hydrolysis directly related to the amounts of competing mEGF. In the direct non-competitive test, ELISA can detect a minimum of 25 pg mEGF, apparently due to the requirement of a longer coating time for smaller amounts and the corresponding loss of immunoreactivity. In the indirect, competitive test, ELISA detects as few as 12 pg, with 100 pg on the plate.

Significance to Biomedical Research and the Program: The isolation of homologous epidermal growth factors from the tissues of mice (mEGF) and humans (hEGF, urogastrone) strongly suggests an important role for these polypeptides in mammalian biology. Development of ultrasensitive assays for the study of these factors is essential to the delineation of their role in influencing normal and abnormal cellular proliferation and differentiation, particularly their potential in diseases such as cancer. Currently, the one sensitive assay published for mEGF is an RIA employing I^{125}-labeled mEGF. It can detect 30 pg in competition. Refinement of the USERIA for mEGF could mean an increase in sensitivity magnitudes above present capability. This would allow the measure of secretion or absorption of mEGF by relatively small numbers of cells, perhaps the activity of primary foci of altered populations. Compared to existing methods, the assay is potentially more sensitive, less expensive, faster and safer to conduct.

Proposed Course of the Project: Continuation of effort to optimize conditions for competitive USERIA; competitive measure of known amounts of mEGF added to sera, urine, clarified cellular lysates and culture medium; generation of primary cultures of mouse salivary glands; initiation of procedures for USERIA measurement of hEGF; initiation of studies to compare polyclonal and monoclonal antibody to hEGF.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05157-01-LEP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical factors that contribute to organ-specific carcinogenesis and selectively modify the susceptibility of fetal tissues to carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. M. Rice	Section Chief	LEP, NCI
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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Factors that modify tissue responses to chemical carcinogens in different organ systems are studied to evaluate their contribution to changing susceptibilities to carcinogenesis in certain tissues during prenatal and postnatal development. Tissues and organ systems currently under study include the nervous system, kidneys, liver, and intestinal mucosa. Modifying processes now being studied include excision repair of DNA, transport of carcinogen into and within cells, and the changing cellular susceptibility to carcinogens during different stages of the cell cycle. Factors investigated in the past, and anticipated for future study, include immune responses to tumors, the induction and genetics of enzymes metabolizing carcinogens, and tumor promotion.

Objectives: To define the roles of biochemical processes that significantly modify the susceptibility of fetal and selected adult organs to chemical carcinogens. Current specific objectives include the following: To investigate the development of capacity for excision repair of DNA in fetal tissues including brain and liver during the course of intrauterine development and to evaluate the role of repair capacity or its absence in high prenatal susceptibility to tumorigenesis in these organ systems in different species. To evaluate the role of the enzyme gamma glutamyltranspeptidase (GGT) in the pronounced organ selectivity of the carcinogen azaserine and related substances which are selectively toxic and carcinogenic for organs such as the kidney and pancreas that are characteristically high in levels of GGT. To evaluate the extent to which susceptibility of a specific target cell, the hepatocyte, varies in susceptibility to chemical carcinogens during different stages of the cell cycle, a factor of major importance in understanding the intrinsically high susceptibility to chemical carcinogens of fetal tissues that have high rates of cell division.

Methods employed: For studies of active transport phenomena involving GGT, cells possessing this enzyme and derivative or mutant lines which do not are grown in cell culture, and the effects of toxic substrates of the enzymes are evaluated morphologically. Uptake and distribution of toxic compounds is determined by liquid scintillation counting of radioactive preparations. Enzyme activities are demonstrated histochemically and quantitated biochemically through standard procedures involving the generation of colored enzyme reaction products. Nucleic acid repair in organs taken directly from fetal tissues is accomplished through alkaline elution procedures without radiolabeling of the target tissues. Nucleic acids are quantitated by different fluorescence emission spectra of bound and non-bound ethidium bromide. Pregnant animals are treated with various doses of carcinogens at precisely defined times during gestation, and at selected intervals after treatment fetal organs are dissected under a microscope and dissociated for study by elution system. The cell cycle kinetics of the regenerating rat liver are determined after subjecting F344 rats to a two-thirds partial hepatectomy, by pulse labeling the regenerating liver with thymidine, quantitating the incorporation of this DNA precursor by liquid scintillation counting of isolated DNA, and by evaluating the proportions of cells undergoing DNA synthesis and in mitosis through high resolution autoradiographic techniques. Rats are injected via the portal vein with selected doses of direct-acting carcinogens at precisely defined times after partial hepatectomy and the carcinogenic response, as well as the evolution of preneoplastic lesions, is determined as a function of time elapsed since partial hepatectomy and as a function of the stage of the cell cycle at which carcinogen treatment took place. Increased synchrony in regenerating liver is achieved by post-hepatectomy injection with hydrocortisone hemisuccinate at 5 hour intervals for a total of 3 injections.

Major findings: Previous reports have documented that for a number of cell lines, sensitivity to toxicity by the carcinogen L-azaserine correlates positively with intracellular levels of GGT. We have extended these studies to investigate whether inhibitors of this enzyme will inhibit azaserine toxicity as well. Cultures of TuWi, an established cell line derived from a human

nephroblastoma, which is high in GGT and very sensitive to the azoamino acid, were treated with azaserine (100 µg/ml) plus one of a series of amino acids (20 mM) in medium lacking L-glutamine. Toxicity was monitored by cell counts on successive days following treatment. L-glutamine, L-methionine, and L-ethionine were found to be highly effective inhibitors of toxicity. Increasing concentrations of these amino acids, up to 100 mM, gave even greater protection. Other amino acids, including L-aspartic acid, L-glutamic acid, L-serine, and L-proline provided little or no protection even at high concentrations. The three protective amino acids were next assayed for their capacity to function as substrates for GGT, i.e., their capacity to accept transfer of the gamma-glutamyl moiety from L-gamma-glutamyl-p-nitroanilide cleaved by GGT. All three of the protective amino acids demonstrated high GGT substrate activity at 20 mM concentration. Amino acids shown to have no inhibitory effects on azaserine toxicity had little or no GGT substrate activity. These findings support the hypothesis that GGT activity is an important factor in determining cellular susceptibility to azaserine toxicity.

In order to study the susceptibility and repair capacity for DNA damage due to alkylating chemical carcinogens in different organs during embryonic and fetal development, a very sensitive method for the evaluation of DNA strain breakage in mammalian cells is essential. Fractionation of DNA by alkaline elution (Kohn, et al. 1976, Biochemistry 15: 4629) was adapted for this purpose. While investigators commonly use radioactively prelabled DNA to quantitate the small amount of DNA in each fraction, the radioactivity itself contributes to strand breakage, and to prelabel experimental animals (pregnant rats or mice) with thymidine-H^3 sufficiently to label DNA adequately in fetal organs is nearly impossible, prohibitively expensive, and inevitably would contribute a significant degree of complicating damage. Accordingly, a simple, reproducible method has been developed to detect the amount of DNA eluted from columns by measuring enhanced fluorescence of ethidium bromide when bound to DNA. Details of the method have been documented in previous reports.

A quantitative excision repair study was conducted in order to explore the relationship between DNA repair activity and organotropic differences in fetal susceptibility to chemical carcinogenesis. Pregnant rats received a single dose of ethylnitrosourea on the 14th day of gestation. Cell suspensions from fetal brain and liver were subjected to DNA fragmentation analysis as a function of time after exposure to the carcinogen by the alkaline elution technique. Increases in elution rate have been shown to be related to the size of single stranded DNA fragments, with size inversely proportional to rate of elution. Repair of alkylation sites resulting from exposure to ENU involves sequential formation of apurinic or apyrimidinic sites in DNA, followed by single strand breakage and rejoining of the break, or alternatively, enzymic cleavage of the DNA strand in which damage has occurred at some point of variable distance from the break, removal of DNA containing the damage, resynthesis and rejoining of the two resulting breaks. Dose dependence of enhanced DNA elution was studied 4 hours after intravenous injection of pregnant rats. Over the dosage range 0.63 mmole/kg to 0.25 mmole/kg body weight, the amount of DNA eluted from liver was slightly greater than from brain, suggesting enhanced breakage. At higher doses of 0.5 and 1.0 mmole/kg, the amount of DNA

eluted from liver was much more than from brain. The kinetics of single strand breakage and rejoining were examined at the lower of these two doses, 0.5 mmole/kg. In fetal rat liver, substantially increased quantities of eluted DNA were observed two hours after treatment. DNA strand breakage as indicated by elutable DNA was seen 4 hours after breakage. At 9 hours after treatment, elution rates approached those of control nucleic acids indicating completion of the repair process. Interestingly, a similar pattern but with much smaller amplitude at the time of maximum elution was observed with DNA from fetal brain. These studies are continuing in order to place these results in the context of alkaline sucrose gradient studies and O^6-alkylguanine excision studies previously reported from this laboratory and from other laboratories which suggest a significant deficit in nucleic acid excision repair capacity in the rat brain, especially during late fetal life when susceptibility to chemical carcinogens is maximal.

Preliminary studies on the effects of chemical carcinogens on the regenerating rat liver indicate that, in regenerating, synchronized hepatocyte populations, susceptibility to neoplastic transformation by the alkylating agent methylnitrosourea (MNU) is greatest in cells that are actively synthesizing DNA, and thus that the S phase of the cell cycle, especially its earlier portion, is a period during which there is markedly enhanced intrinsic cellular vulnerability to neoplastic transformation. This conclusion was drawn from studies in which carcinogen was administered to partially hepatectomized rats at 7 distinct intervals after partial hepatectomy, and constitutes the most extensive study of cell cycle-dependent carcinogenesis ever attempted in vivo. The conclusions are especially convincing since the onset of S could be delayed by as much as 10 hours by administering the hormone hydrocortisone by intraperitoneal injection at intervals after partial hepatectomy, resulting not only in a delay in onset of S, but improved synchrony once decay of the block allowed the cell cycle to proceed in the regenerating liver. Delay of 10 hours in the onset of S delayed the peak tumor incidence in carcinogen treated rats by the same interval. However, use of the carcinogen MNU, despite its independence of metabolic factors for activation, proved to be less than perfectly adapted for the purposes of these experiments, since a large proportion of the total injected dose escaped from the liver even though injected via the portal vein. This led to a high incidence of lethal tumors with relatively short latency periods in other critical organ systems, including the nervous system, kidneys, intestines, Zymbal's gland, and odontogenic tissue. These complicating extrahepatic tumors were most generally the cause of death in experimental rats, and effectively determined the duration of the period of observation that was possible in the carcinogenesis phase of these studies. Preliminary experiments with methyl(acetoxymethyl)nitrosamine, DMN-OAc, an ester of the presumed reactive metabolite of dimethylnitrosamine, showed that this compound, which which is dependent on esterase for its activation rather than any oxidative enzyme system, resulted in a higher incidence of hepatocellular tumors than MNU and caused practically no tumors in other organ systems when injected into partially hepatectomized rats via the portal vein. Subsequent large scale experiments, now in progress, indicate that this is the ideal agent to use for cell cycle studies, since levels of esterases do not change over the period of

hepatectomy and regeneration in liver and labeling of DNA by DMN-OAc-C^{14} is essentially constant irrespective of the stage of the cell cycle in regenerating liver when the labeled carcinogen was given.

Significance to biomedical research and the program of the Institute: The biochemical basis of the well established high fetal susceptibility to chemical carcinogens remains poorly understood. The fetus is clearly at greatly elevated risk from exposure to chemical carcinogens, a fact that must be considered in estimations of relative human risk from environmental exposure to such agents. The factors that contribute to this enhanced susceptibility remain to be adequately evaluated. The role of DNA repair processes, best shown in the enhanced susceptibility to UV carcinogenesis in individuals deficient in such repair as a result of the hereditary condition xeroderma pigmentosum, indicates that repair is significant in controlling the consequences of damage to cells inflicted by carcinogenic agents, but the development of this capacity during prenatal life has been very little studied. It could easily contribute in part to the susceptibility of fetal tissues which may be deficient in activity or fidelity of DNA repair.

Organ specificity in carcinogenesis by different classes of agents is of obvious importance in the problem of extrapolating between species and in understanding the spectrum of tumors ascribed to environmental causes in human beings. Most such studies in this area have concentrated on capacity of target cells to metabolize carcinogens to ultimate reactive forms. The means by which carcinogens penetrate cells have received little attention in comparison. In particular, the concept that certain agents may be actively transported into target cells has not been investigated in the context of carcinogenesis. The first indication that such a phenomenon may in fact exist is afforded by the studies on GGT, which therefore are important as a potential model for active transport in relation to carcinogenesis.

It has long been known that cells in an active mitotic state are more intrinsically susceptible to chemical carcinogens than post-mitotic cells or cells that are not cycling. The exact reasons for this are not clear; a reasonable hypothesis is that DNA may be more vulnerable to damage in certain phases of the cell cycle, and that in cycling cells, there is a greater probability that DNA repair will not be completed before programmed DNA synthesis encounters a defective portion of the genome bearing a carcinogen induced lesion. Whether in fact cells are especially vulnerable to carcinogens at a particular stage of the cell cycle is thus of importance not only for perinatal carcinogenesis, but for carcinogenesis in adult tissues where mitotic activity is high, including various lining epithelia such as intestinal mucosa which constitute major sites of important neoplasms of man.

Proposed course: The prenatal development of capacity for excision repair of DNA in liver, brain, and other tissues will continue to be studied by high-resolution alkaline elution, in a continuing effort to titrate physical repair of single strand breaks at dosages of simple monofunctional methylating and ethylating agents below cytotoxic levels, as judged from histological sections of organs of interest. This procedure should allow for greater resolution

than the relatively crude alkaline sucrose gradients initially employed, will eliminate artifacts due to radioactive decay of tritium labels and the severe selection process of short term in vitro cultivation for purposes of labeling, and should allow meaningful comparison of species of experimental animals that differ greatly in prenatal susceptibility to carcinogens in various organ systems. If the procedure is successful with these agents, carcinogens with more complex chemical structures will be studied in the same fashion to determine which types of damage are repairable by fetal tissues, and how these correlate with prenatal susceptibility to carcinogenesis in different organ systems in different species.

Continuation of the studies of the role of GGT in active transport of chemical carcinogens will focus on determining whether the phenomena observed with azaserine are applicable to other carcinogens. If the phenomenon is found to apply to other compounds of major interest, the studies on this system will be continued; if not, studies on the biochemical basis for organotropism of azaserine will be brought to an orderly conclusion and the project terminated.

Studies on the role of the cycle in susceptibility to carcinogenesis and possibly other forms of genotoxic damage including mutation, will be continued. Techniques developed in the course of these studies, including characterization of the biology and morphology of liver cell tumors in rats resulting from a single transient exposure to an alkylating agent, will be applied elsewhere in the program of this Section. We plan to attempt to resolve apparent discrepancies between capacities for DNA repair in fetal liver and brain in rats and mice, and the distinct differences in susceptibility to oncogenesis in these species. Prenatal susceptibilities to hepatocarcinogenesis at variance with studies of repair in the fetal and postnatal nervous system and liver in both species. One approach to this problem will be through studies of tumor promotion in the liver. It is well known that agents such as phenobarbital are capable of acting as classic tumor promoters on hepatocytes in both the rat and the mouse. Experiments will be conducted to determine whether postnatal promotion by phenobarbital will promote expression of latent neoplastic hepatocytes in the livers of rats transplacentally exposed to alkylating agents such as ENU, which in the absence of such postnatal promotion would never proliferate to form tumors. In addition, the fact that liver cell tumors are inducible not only in the mouse, but in sub-human primates by prenatal exposure to alkylating agents, invites the utilization of phenobarbital in transplacental carcinogenesis studies in primates to demonstrate promotion phenomena in a primate species in an experimental context for the first time. This would be a major contribution to the available data base on which extrapolation between species of the effects of tumor promoters could be based, which at the present time is restricted entirely to rodents and therefore entirely hypothetical as a significant category of human risk.

Finally, the role of fetal metabolism versus maternal metabolism as a determining factor in the capacity of metabolism-dependent carcinogens to act as transplacental carcinogens has in the past been studied in this Section indirectly, through studies of the biological effects of agents whose metabolic paths are known. The recent literature on metabolism of foreign compounds,

however, suggests that conclusions on the role of fetal metabolism based entirely on responses to polynuclear aromatic hydrocarbons may not be applicable, and in fact may run counter to, the effects of other important classes of carcinogens including the nitrosamines. Direct study of this phenomenon in both biochemical and genetic contexts is planned as a future activity of this Section, and is dependent on recruitment of a scientist experienced in techniques for studying carcinogen metabolism in the laboratory to participate in these experiments.

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SUMMARY REPORT

LABORATORY OF MOLECULAR CARCINOGENESIS

October 1, 1979 through September 30, 1980

"Plans, develops, and conducts a research program designed to (1) clarify the molecular biology of carcinogenesis; (2) elucidate the fundamental nature of the interactions of carcinogenic agents, especially chemical, with biological systems in the induction of cancer; (3) define those environmental and endogenous factors which relate to and modify the carcinogenic process; and (4) clarify the metabolic regulatory processes which are related to carcinogenesis."

The goal of the Laboratory of Molecular Carcinogenesis is to understand the molecular basis of carcinogenesis with the view toward identifying susceptible populations and preventing human cancer. The research program is designed to understand the molecular basis by which carcinogenic agents cause malignant transformation and identify and characterize those exogenous and endogenous factors involved in carcinogenesis. The laboratory seeks to clarify the metabolic interaction of exogenous and endogenous agents in the living organism at the molecular, cellular and organism levels and seeks to understand the consequences of these interactions in terms of cell regulation and carcinogenesis. These processes are studied in biological preparations and cells from experimental animals and humans.

Cancer is a disease in which the genetic information and/or expression is altered. This altered phenotypic expression may be due to genetic or epigenetic events induced by either xenobiotic or endogenous factors which result in altered patterns of gene control. The central aim of the Laboratory is to understand how exogenous carcinogens and endogenous factors are processed by enzymatic mechanisms, how the carcinogen is converted to active forms and the nature of the interaction between the latter and the gene action system. Systems involved in the repair of genetic damage induced by carcinogens are studied. Understanding is sought on how the interaction with carcinogens results in the modifications which characterize malignant transformation. The aim of these studies is modification, elimination or prophylaxis of the carcinogenic response in the human population.

Cell Genetics Section - *"Studies (1) the process of malignant transformation of cells by chemical carcinogens; (2) the genes and gene products responsible for the transformed state of the malignant cells."* The aim is to understand the molecular events governing the malignant transformation of cells by chemical and physical carcinogens. In order to achieve this goal, mainly the following approaches have been taken; first, development and improvement of experimental systems such as an assay system for cell transformation, mutation, and lymphoblastoid cell survival, and isolation of cell mutants and variants; secondly, investigation of the initiation process of transformation by carcinogens; and thirdly, search for the molecules responsible for the transformed phenotype. Current primary areas of research in this section are: 1) Development of a quantitative system for simultaneous

assay of malignant transformation and mutation of cultured cells including human cells by chemical carcinogens and irradiation; 2) Investigation of the genetic and physiological factors affecting cell transformation with special attention to the DNA repair function; 3) Cellular and molecular effects of psoralen plus ultraviolet light; and 4) Attempts to identify the cellular genes and gene products involved in the expression of the transformed phenotype.

In order to study the mechanism of cell transformation, to obtain information about how to extrapolate the results obtained from the carcinogenesis experiments in animals and many in vitro assay systems into human risk, and to compare the mutation and transformation, it is useful to have a reliable, rapid and quantitative system for simultaneous assays of malignant transformation and mutation in the cells of different species and origins including human cells. The replating procedure was introduced into the transformation assay and any optimum number of cell generations after carcinogen treatment rather than the time allowed for expression was determined to obtain the maximum mutation frequency. Thus combined with the previous observations in our laboratory, it became possible to assay the induction of mutation and transformation under the same condition of expression as well as the condition of exposure to carcinogens. The search for the selective condition or biological indicator for the chemically transformed human fibroblast cells indicates that the ability of the cells to grow over the monolayer sheet of untransformed cells is the clearest and most objective marker of the transformed cells among indicators tested and that this property could be used as an efficient condition of selective growth of the transformed cells.

As one of the approaches to identify the genetic factors affecting cell transformation, human cancer-prone genetic diseases such as xeroderma pigmentosum (XP), ataxia telangectasia (AT), familial malignant melanoma with dysplastic nevi (DNS), and neurofibromatosis (NF) are being studied with special attention to the relation of DNA repair defects and clinical features. A semi-automated technique to facilitate the autoradiographic measurement of DNA repair in XP cells has been developed. AT lymphoblastoid cells were found to be abnormally sensitive to bleomycin measured in terms of cell killing and in induced chromosomal breaks. Studies of the level of aryl hydrocarbon hydroxylase (AHH) in leukocytes of patients with psoriasis suggest that there is a specific AHH elevation in lymphocytes but not in monocytes.

Another approach to the genetic factors is to characterize the cell variants of different susceptibility to the transformation which have been isolated from A31-1 clone of Balb/3T3 cell lines in our laboratory. No differences were observed between these cell variants in the sensitivity to the induction of ouabain-resistance mutation by UV and in the activity of excision repair of benzo(a)pyrene (BP)-induced DNA damage. It was found that all these cell variants have a poor ability to excise BP-DNA base adduct which are much like the cells from XP patients with respect to repair deficiency. Studies on the effect of growth condition of A31-1-1 cells after UV-radiation revealed that there are differences in the requirement of post-UV cell generation for expression between induction of transformation and mutation. Holding A31-1-1 cells at nongrowing state after UV resulted in the gradual loss of the abilities of the cells to be transformed but not those to be mutated.

Psoralen plus long wavelength ultraviolet radiation (UV-A) are being investigated as a model system for clinically relevant photochemical carcinogenesis. This therapy is used experimentally in treatment of psoriasis but has been found to be carcinogenic in mice and humans. 8-Methoxypsoralen (8-MOP) and UV-A treatment resulted in decreased DNA synthesis, DNA-8-MOP cross-link formation, cell killing, increased sister chromatid exchanges and decreased mixed leukocyte reactivity. An immunoassay to detect antibodies against 8-MOP-DNA in patients and rabbits is being attempted.

Attempts to detect, isolate and identify the cellular genes involved in the expression of transformed phenotype are being made using the methods developed in the field of somatic cell genetics such as cell hybridization, gene transfer, and gene cloning techniques. One approach is to detect the cellular genes which have the capacity to induce or suppress the transformed phenotype. The hybrid cells between normal human diploid fibroblasts and chemically transformed counterparts showed a transformed phenotype in vitro. This observation indicates that genes isolated from transformed cells may be able to induce the transformed phenotype if they are successfully transferred into normal cells. Exposing normal human diploid fibroblasts to DNA extracted from ouabain-resistant human fibroblasts resulted in the appearance of ouabain-resistant cells. These results suggest that DNA-mediated transfer of the transformed phenotype into normal diploid human cells may be possible and that cellular genes responsible for the expression of transformed phenotype can be identified by using this cell system.

Another approach is to clarify the structure and mechanism of expression of the genes coding macromolecules, alterations of which have been associated with the expression of transformed phenotype. Actin, a major protein in the cytoskeleton, has been considered to be involved in many cellular functions including expression of transformed phenotype and its altered molecular form has been found in chemically transformed human fibroblasts. The results with in vitro translation of mRNA extracted from the transformed cells indicate that alteration of actin molecule is not due to the post-translation modification. cDNA synthesized from actin mRNA of normal and transformed human cells was inserted into plasmid pBR322 DNA. Characterization of cloned cDNA specific for actin mRNA and isolation of genomic actin gene by using cloned cDNA are currently being made.

Metabolic Control Section - *"Studies (1) the metabolic effects on tissues and cells resulting from their exposure of chemical carcinogens and the relationship between these effects and malignant transformation; (2) the metabolic activation and detoxification of the polycyclic hydrocarbons and the relationship of this metabolism of individual susceptibility."* This section studies the molecular events of malignant transformation induced by chemical carcinogens, in particular those of the polycyclic hydrocarbon class. The aim is to understand the enzymatic conversion of carcinogens to either detoxification forms or to the active carcinogenic metabolite. During evolution humans have been exposed to foreign chemical compounds (including carcinogens) and have developed systems for their detoxification and elimination. These systems primarily involve microsomal cytochrome P-450 mixed-function oxygenases, but also include epoxide hydratases and conjugating enzymes. The vast majority of foreign compounds are processed

by these enzyme systems. The mixed-function oxygenases are influenced by a variety of environmental factors such as drugs, pesticides or carcinogens, and are influenced by the nutritional and hormonal state of the animal. The age, sex and genetic makeup also determine enzyme activity. Work in this laboratory provided the key studies which showed this enzyme system to be responsible for the activation of procarcinogens to what may be their ultimate carcinogenic form.

A primary goal is to define the enzymatic mechanism by which polycyclic hydrocarbons are either activated to carcinogenic forms or to detoxified products. As these enzymes are characterized and sensitive methods are developed for their assay, it may be possible to characterize an individual's enzymatic makeup with respect to carcinogen metabolism and to understand the relationship between this metabolism and individual susceptibility to polycyclic hydrocarbon carcinogenesis. The known sequence of PCH metabolism is as follows: The first step is oxygenation by the microsomal mixed-function oxygenases one indicator of which is aryl hydrocarbon hydroxylase (AHH). Very sensitive methods have been developed for the latter which are applicable to human tissues. Secondly, epoxides are hydrated to dihydrodiols by oxide hydratase. The oxides are also metabolized by glutathione S-transferases. The phenols and dihydrodiols are conjugated by UDP glucuronic acid Sensitive assays for each of these systems as well as a method for total metabolite analyses by high pressure liquid chromatography (HPLC) have been developed.

The approach is to identify and fully characterize the enzymes responsible for carcinogen activation and metabolism. This knowledge may be important for understanding the role of biochemical individuality in carcinogenesis. We plan to assess the types and amounts of these enzymes in a human population using both metabolic and immunological approaches. The latter will include monoclonal antibodies. In addition we seek to understand the molecular biology and regulation both at the genetic and epigenetic levels of the carcinogen metabolizing enzyme systems. This approach will include techniques of DNA recombination and DNA sequencing.

We earlier introduced the use of high pressure liquid chromatography for the analysis of polycyclic aromatic hydrocarbon metabolism. We are continuing this development in an attempt to improve the separation efficiency and reproducibility. We have found that the addition of anti-oxidents prevents oxidation of low levels of metabolites and we have used this improvement to analyze low levels of metabolism in human cells.

We have taken a completely new and very promising approach to biochemical individuality in human carcinogenesis. We plan to apply the use of monoclonal antibody tools to probe the types and profiles of human carcinogen metabolizing enzymes in different individuals. As a first step we have successfully prepared monoclonal antibodies to a purified rabbit liver cytochrome P-450. This monoclonal antibody is highly specific and does not cross react with any of the other cytochrome P-450 from rabbit liver.

Another key enzyme involved in carcinogen metabolism is epoxide hydratase. A rather important question has been the role of this enzyme in the detoxification of the active carcinogenic forms of BP. We have found that in

a variety of conditions the diol epoxides are susceptible to the enzymatic action of the epoxide hydratases, thus establishing a role for this enzyme in carcinogen deactivation.

Human lymphocytes and monocytes have been shown to exhibit mixed-function oxidase activity, a property which in many tissues and species is responsible for the activation of polycyclic hydrocarbon carcinogens. The levels of this activity vary among individuals and may be enhanced by culturing the cells with various inducers. In rat and rabbit tissues cytochrome P-450 is present in multiple forms and these forms differ in their substrate specificities. Multiple forms of cytochrome P-450 have not yet clearly been demonstrated in human tissues. Antibodies prepared against two forms of rat liver cytochrome P-450 have been found to inhibit mixed-function oxidase (i.e., benzo(a)pyrene hydroxylase) activity of human lymphocytes and monocytes. In this study the specificity of this inhibition is being investigated further with special attention being given to determining the relationship between antibody binding to cytochrome P-450 and inhibition of enzyme activity.

Nucleic Acids Section - *"Studies (1) interaction of chemical carcinogens and their analogs with nucleic acids and the action of cellular processes regulating nucleic acid metabolism; and (2) the effect of these agents on the subcellular organization of nucleic acid macromolecules."* The primary areas of research in this section are: 1) an investigation of the mechanism of DNA repair in normal and malignant cells in order to understand the relationship of DNA damage and its repair to human cancer; 2) determination of the structural-functional relationships of DNA modified with chemical carcinogens; and 3) the possible role that misreading of aminoacyl-tRNAs during protein synthesis may have in the carcinogenesis and evolutionary changes in aminoacyl-tRNA (anti-codon): codon and tRNA:aminoacyl-tRNA synthetase interactions. A group of fourteen of seventy human tumor cell strains has been identified as deficient in repairing methylation damage. Thirty-three strains of normal human fibroblasts were proficient in such repair, including two fibroblast strains prepared from people whose tumors gave rise to repair deficient strains. Such repair deficient cells were killed by low concentrations of alkylating agents, including chemotherapeutic agents, repaired alkylated infecting viruses poorly and failed to remove O^6-methylguanine from their DNA after methylation treatment. Strains both deficient and proficient in the repair of methylation damage were found to incise their methylated DNA, but only the repair-proficient strains could restore their DNA to normal conformation.

Human tumor cells were found less able than human diploid fibroblasts to discriminate against stably incorporating the nucleoside analog BudR into their DNA. Human tumor cells and human diploid fibroblasts were shown to use distinquishable mechanisms for synthesizing their DNA.

Novobiocin was found to block the DNA synthesis that accompanies repair of UV or MNNG damage. Upon removal of the novobiocin from cultures blocked in repair, a burst of repair synthesis was observed, consistent with biological data indicating that novobiocin does not enhance UV or MNNG produced cell killing when present with the UV or MNNG treated cells for periods up to a day after the toxic treatment.

Antibodies against DNA altered by reaction with benzo(a)pyrene diol-epoxide I (BPDE-I) were shown to react with that DNA but not with native or denatured DNA which had received no treatment, or with protein or RNA which had received such treatment. These antibodies were used in fluorescent-straining studies of DNA repair by BPDE-I treated cells. Initial results indicate that such repair may be observable using this technique.

Attempts to establish sensitive methods for detecting structural and biological modifications of modified DNAs have resulted in an assay in which a DNA plasmid is treated with BPDE-I and analyzed for induced mutation production and survival as a function of the number of BP molecules bound per DNA molecule. DNA sequence comparisons of wild-type with mutant DNAs should establish the etiology of the mutations produced. In vitro studies showed that DNA replication did not proceed past a DNA bound BP molecule.

A method utilizing two dimensional gel electrophoresis was employed to separate supercoiled DNAs having equal numbers of positive and negative supercoils. This method also allowed the direct determination of the superhelical density of the supercoiled DNAs studied. Binding constants and thermodynamic parameters were determined from measurements made under various experimental conditions.

The method was also applicable to analyzing the number of psoralen plus near UV produced crosslinks in DNA. Intercalation of DNA by a number of BP metabolites was studied. Successful intercalation occurred maximally when such molecules were hydroxylated at the 7 and 8 positions.

An isoacceptor of Lys-tRNA, which others have correlated with cellular division, was used less frequently in protein synthesis than other lysine isoacceptors with the same codon recognition properties.

Patterns of codon recognition of isoacceptor aa-tRNAs fractionated from P. physarum were determined. Somewhat surprisingly, such patterns were more similar to those observed in the animal kindgom (hymenoptera, mammals) than those in the plant kingdom (wheat germ).

Cross-aminoacylation studies with tRNAs fractionated from E.coli, yeast, and wheat germ using synthetases from these organisms have identified several heterologous cases in which complete aminoacylation of one isoacceptor and little or no aminoacylation of another occurred. No examples of efficient misrecognition were observed.

Protein Section - *"Studies (1) the relationship of proteins to the carcinogenic process; (2) interactions between carcinogenic agents and proteins; (3) changes in proteins occurring as a result of or concomitant with the administration of carcinogenic agents; relates the genetic control of such proteins to inheritance in laboratory animals and man."* Studies on the interaction of carcinogens with chromosomes and their constituents were initiated in the last year. It was found that benzo(a)pyrene 7,8-diol 9,10-epoxide I binds to polytene chromosomes. The binding pattern was compared with the banding pattern of the chromosome, from which it was concluded that

at this level of observation carcinogen bound to the chromosome in a random manner. Methodology for extending these studies to nucleosomes and specific DNA sequences has been developed and may provide increasingly detailed information as to the binding pattern of various carcinogens. There has also been significant progress in the definition of the role of chromosomal proteins in maintaining the structure and regulating the function of chromosomes. It has been found that microinjection of anti-histone antibodies into oocytes of the salamander resulted in the retraction of the transcription loops. The antigen-antibody complex thus further inhibits progress of RNA polymerase along the template, thereby causing premature release of transcripts. There have been additional improvements in immunochemical methods for studying the chromatin subunit. Antisera specific to purified histone fractions have been used to assess the antigenicity of histones present in chromatin derived from various sources.

It was found that the antigenicity of histones was similar in the several sources, and markedly diminished compared to the antigenicity of histones free in solution. It was concluded that in addition to chemical similarity among these constituents the histone-DNA contacts in the chromatin subunit are very similar, suggesting evolutionary stability for both the constituents and the structure. Antibodies have been elicited in rabbits using a nucleosome purified from chicken erythrocyte nuclei and extensively characterized. It was concluded that the antibodies were directed against nucleoprotein antigenic determinants composed of the N-terminal portion of histones and segments of DNA.

These studies have been facilitated by the development of a solid-phase radioimmunoassay. Among other findings using this assay were observations that the binding of protein HMG-1 to nucleosomes reduces the reaction with anti-HMG-1, probably a reflection of conformational changes resulting from the binding. Similarly, a portion of the antigenic determinants of histones complexed in nucleosomes is also not available for reaction with antibodies.

Nucleosome-antibody interactions were studied by computer graphics. Among the interesting findings from this endeavour was the suggestion that in the large majority of cases immunoelectron microscopic observations will fail to resolve the contour of an antibody attached to a nucleosome unless a secondary tag is added.

Electrophoretic and immunological studies have demonstrated that the midge Chironomus thummi contains a protein antigenically very similar to the mammalian chromosomal protein HMG-1. Immunofluorescence studies show that this protein was present in both the band and interband regions of the chromosome and that the location of the protein changes during the development of the larvae.

It was reported last year that antibodies prepared against 2 forms of rat liver cytochrome P-450 inhibited cytochrome P-450-dependent BP hydroxylation by human lymphocytes and monocytes. The initial findings that the enzymatic activity in lymphocytes was more completely inhibited than the enzymatic activity in monocytes was confirmed in additional studies on 8

human subjects. The extent of inhibition varied somewhat among individuals, but there was no correlation with either the initial BP hydroxylase activity of the cells or the extent to which the activity increased upon induction. Experiments to identify the binding sites within the cells have so far been frustrated because of low levels of the enzyme in the cells and a significant amount of non-specific binding. Continued studies on the electrophoretic discrimination of different forms of cytochrome P-450 in monocytes have been facilitated by the discovery that significant purification of this enzyme by anion exchange column chromatography leads to improved results. The SDS-PAGE protein banding patterns of such preparations were not changed upon induction, a result which contrasts with the clearly defined differences observed in control and induced rat liver. With isoelectric focusing the enzymatic activity from BA-induced monocytes was found in a broad band having a pI of 4.3-5.5, a range which overlaps pI values found for induced and uninduced preparations for liver.

In the course of characterizing milk proteins from human and murine sources, a highly phosphorylated glycoprotein of approximately 50,000 molecular weight has been observed in the colostral milk of mice and in the milk of humans. These highly modified proteins, somewhat alike in the 2 species, may have escaped previous detection because, though they are readily observed using the specific stains developed here, earlier standard techniques are inadequate. Further characterization of these proteins is in progress.

Studies on the size distribution of hnRNA molecules in normal and malignant cells have progressed sufficiently far to permit the conclusion that conversion to malignancy by chemical or UV treatment of mouse and human fibroblasts is accompanied by very little, if any, change in size distribution. There were, however, significant differences in hnRNA distribution between species. This latter observation contrasts with the very close similarity observed for other types of RNA in the mammalian species.

Experimental difficulties in obtaining undegraded and uncontaminated species of hnRNA for analysis have required continuous small improvements in the methods for preparation of RNA, especially the removal of contaminant ribonuclease from reagents. Some progress has been achieved in this area. Extensive improvements in the computer programs used in analysis of thermal degradation data have been achieved. The final results achieved by the improved programs do not differ significantly from earlier programs, but require both substantially less knowledge and experience on the part of the user and significantly reduced computer time for solution.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04496-03 LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

In situ Organization of Chromosomal Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Department of Biochemistry, University of Oregon, Corvallis, OR
Department of Biochemistry, Kansas State University, Manhattan, KS

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.9	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies specific to histones, non-histone chromosomal proteins HMG-1, HMG-2 and HMG-17 and to nucleosomes have been used to study the structure-function relation of defined chromosomal components in chromatin and chromosomes. A sensitive solid-phase radioimmune assay for chromosomal components has been developed. The interaction of IgG molecules with the chromatin subunit has been studied by the interactive computer graphics technique. The antibody molecule cannot interpenetrate between adjacent DNA strands to reach determinants in the histone regions covered by DNA. The complexing of chromosomal proteins into the nucleosomal conformation brings about conformational changes or steric hindrance so that part of the antigenic sites are masked. Nucleosomes elicit antibodies directed against determinants which are nucleoproteins composed of DNA and the N-terminal segments of the histones. Specific antibodies have been microinjected into oocytes of Pleurodeles waltlii. Antibodies to histones bring about a specific retraction of the transcriptional loops in the lampbrush chromosomes present in these oocytes.

Cooperating Units (continued):
Division of Membrane Biology, German Cancer Research Center, Heidelberg, Fed. Rep. Germany

Project Description

Objectives: To understand the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes. To explore the possibility that neoplastic transformation is associated with defined alterations in either the type or the organization of chromosomal components.

Background Information and Research Strategy: The chromatin fiber, which is the backbone of chromatin and chromosomes, is a dynamic structure. Thus, it is difficult to determine the structure-function relation of defined chromosomal components. This question was approached by purifying chromosomal proteins, eliciting specific antibodies to these and using the antibodies as probes for studying the in situ arrangement of the defined chromosomal components at various stages of chromatin organization. So far it was possible to purify the antigens, elicit antibodies, and adapt and develop various immunochemical techniques for detecting and quantifying the binding of specific antibodies to chromatin and chromosomes. Immunoelectron microscopy and immunofluorescent techniques are used to study the organization of histones and defined non-histone chromosomal proteins in the interphase, transcribing and replicating chromatin fiber and in metaphase and polytene chromosomes.

In the past year the research effort was concentrated in the following areas: 1) development of new sensitive immunoassays for chromosomal components; 2) studies on immunochemical aspects of the chromatin subunit; 3) studies on the interaction of an IgG molecule with the chromatin subunit by the interactive computer surface graphic approach; 4) studies on the organization of non-histone chromosomal proteins HMG-1 and HMG-17 in polytene chromosomes and in HeLa cells; 5) studies on the feasibility of introducing defined antibodies into cells as a means to elucidate functional aspects of defined chromosomal proteins.

Methods Used: These have been described in the previous report.

Findings: 1) New immunological assays for chromosomal components. Since most chromosomal components do not have an assayable function, immunological techniques can be used to detect and quantify them. A convenient, accurate and sensitive radioimmunoassay for chromosomal components has been developed. Histones, non-histones and nucleosomes can be assayed in the nanogram range at sera dilution higher than 1:1000. As such, this technique is more sensitive and versatile than any other technique previously used to assay chromosomal components. It also can be used to study antigens in their native state.

The method is an adaptation of the solid-phase radioimmune assay. Antigens are added to polyvinyl plates to which they bind very avidly. After blocking

unreacted sites on the plates, antisera is added. The relative amount of IgG bound is determined by adding ^{125}I-protein A and radioactive counting.

Attempts to detect and quantitate defined antigens in cells and nuclei revealed that the antibody binding is specific, but not quantitative. Thus, there was no linear relation between the number of cells or nuclei added to the plate and the amount of antisera bound. Most probably this reflects a situation where the avidity with which the cells bind to the plates is dependent on cell density.

Using the solid-phase radioimmunoassay, it was found that 1) Non-histone chromosomal protein HMG-1 is highly conserved during evolution; 2) Mononucleosomes contain as much HMG-1 as di- and trinucleosomes, suggesting that this protein is not exclusively associated with internucleosomal regions; 3) Chromatin subunits derived from different sources contain similar amounts of HMG-1, suggesting evolutionary conservation of the quantity of this protein in cells; 4) The binding of protein HMG-1 to nucleosome brings about conformational changes or results in steric hindrance so that part of the anti-HMG-1 antibodies cannot bind to their antigenic determinants; 5) Part of the antigenic determinants of the histones complexed in nucleosomes are also not available to interaction with antibodies.

The radioimmunoassay is presently used to follow purification of antigens by standard biochemical techniques. This test is easily converted to an enzyme-linked immunoassay. Antibodies coupled with alkaline phosphatase and the substrate paranitrophenol are most reliable. This immunoassay procedure will eliminate the biological hazards associated with working with radioactive components, will increase the sensitivity of the assay, and will allow further automatization of the immunological assay.

2) Immunochemical aspects of the chromatin subunit. Towards investigating the question of the evolutionary conservation of the chromatin subunit, antisera specific to purified histone fractions have been used to assess the antigenicity of histones present in chromatin derived from various sources. The purified chromatins were reacted with the antisera and the reaction mixture centrifuged. The amount of antibodies remaining in the supernatant was measured by the complement fixation techniques. The results indicate that: 1) The antigenicity of the histones present in chromatin is markedly diminished as compared to the antigenicity of the histones free in solution; 2) The antigenicity of the histones present in chromatin derived from various sources is very similar. It is concluded that: 1) The nucleosomes are very similar immunologically, suggesting evolutionary stability in the histone-DNA contacts in the chromatin subunit; 2) The major reason for diminished antigenicity of the histones in chromatin is that they are complexed in the nucleosome conformation.

The question of histone-DNA contacts in nucleosomes is investigated by two additional serological approaches. One involves spacing of histones in nucleosomes by assessing steric hindrance to sequential antibody addition. Preliminary results indicate that saturation of nucleosomes with one type of anti-histone antibody does not interfere with the binding of another type of

anti-histone antibody. This suggests that the antigenic sites of the histones in nucleosomes are not clustered. Another approach involves labelling the 5'-terminal bases with ^{32}P, adding antibodies to histones and determining the DNAase I cleavage pattern of the nucleosomal DNA. If antibody addition inhibits DNAase I digestion, then this approach will allow precise mapping of histone-DNA binding sites.

Antibodies to the chromatin subunit have been elicited in rabbits by immunizing with nucleosomes purified from chicken erythrocyte nuclei. Specific serological reaction between the antisera and the nucleosomes was detected by immunodiffusion, immunofluorescence, microcomplement fixation, solid-phase radioimmunoassay, immunosedimentation and polyacrylamide gel electrophoresis of 5'-^{32}P-end labelled nucleosomes. The antisera did not react with DNA extracted from nucleosomes, core histones or the cross-linked histone octamer from chick erythrocytes, calf thymus total histones or with chromosomal proteins HMG-1, HMG-2 and HMG-17. Nucleosome antigenicity was not affected by redigestion with micrococcal nuclease. Digestion with proteases resulted in 100% loss of antigenicity while digestion with DNAase I resulted in 50% loss of antigenicity. The antisera reacted well with trimer, dimer, mononucleosomes, and subnucleosomal particles. It reacted better with homologous nucleosomes than with heterologous nucleosomes. It is concluded that the antibodies in the sera are directed against nucleoprotein antigenic determinants composed of the N-terminal portion of histones and segments of DNA.

3) Nucleosome-antibody interactions studied by computer graphics. The binding of an IgG molecule to a chromatin subunit has been simulated and studied by the interactive computer graphics technique. Examination of stereo projections of structures resulting from juxtaposing an IgG molecule next to a nucleosomal DNA reveal the following: 1) The antibody molecule cannot interpenetrate between two adjacent turns of DNA to reach determinants in the histone regions covered by DNA; 2) Within a two-dimensional plane up to 10 molecules of IgG can bind to the circumference of a nucleosome. This suggests lack of steric hindrance to antibody binding; 3) In the large majority of the cases, immunoelectron microscopic observations will fail to resolve the contour of an antibody attached to a nucleosome unless a secondary tag is added. The insights obtained by this approach facilitate a better understanding of the processes associated with the binding of an IgG molecule to the nucleosomal subunit. IgG molecules specific to chemical carcinogens, UV-irradiated DNA and non-histone proteins may bind to the genome in a similar fashion.

4) Organization of non-histone chromosomal proteins HMG-1 and HMG-17 in chromatin and chromosomes. IgG molecules specific to HMG-1 and HMG-17 have been used to study the distribution of these proteins in a nucleosomal gradient. The presence of the proteins in various fractions obtained from the gradient was determined by the solid-phase radioimmunoassay. It was found that HMG-1 was present both at the top of the gradient, unassociated with nucleosomes, as well as in the regions containing various chromatin multimers. Since the amount of HMG-1 in mono-, di- and trinucleosomes was similar, it is concluded that at least part of the HMG-1 is associated with the core particle. Protein HMG-17 was always associated with nucleosomes. Since some of these experiments

were done with chromatin that was extensively digested, it is concluded that the HMG-17 molecules which were released during digestion rebound to intact nucleosomes.

The midge Chironomus thummi contains a protein which is antigenically very similar to mammalian chromosomal protein HMG-1. This was determined by examining a homogenate of nuclei, purified from the salivary gland, by the solid-phase radioimmunoassay. The cross reacting moiety is probably authentic HMG-1. This was determined by electrophoresing chromosomal proteins from the nuclei of salivary glands in SDS-polyacrylamide gel. The proteins were transferred from the gel to DBM paper to which they bind covalently. The DBM paper was treated with anti-HMG-1 and the location of antibody on the paper visualized by treating the paper with ^{125}I-protein A followed by autoradiography. The results indicate that the antigenic moiety had an electrophoretic mobility indistinguishable from that of HMG-1 purified from calf thymus.

This finding opens the way to correlate the location of HMG-1 with various structural and functional features of chromatin which can be conveniently studied using the giant polytene chromosomes found in the salivary glands of Chironomus. Immunofluorescence studies reveal that 1) This protein is not confined to either the band or interband regions of the chromosome; 2) The observed location of the protein changes during the development of the larvae.

Attempts were made to visualize the location of chromosomal protein HMG-17 in eukaryotic cells. HeLa cells, KD cells and V79 cells fixed by a variety of techniques failed to yield fluorescence when examined by the indirect fluorescence technique. Either the amount of HMG-17 in a cell is too low to be detected by this technique or in the "intact" cell the antigenic sites of this protein are not available to antibody binding.

5) Microinjection of functional antibodies. Introduction of functional antibodies into a living cell potentially can lead to the understanding of the cellular role of the respective antigen. Anti-H2B IgG molecules were microinjected into oocyte nuclei of the salamander Pleurodeles waltlii. The oocytes are a convenient experimental system because of their size and because they contain lampbrush chromosomes. These chromosomes contain several thousand highly active genes which can be visualized in the transcribing process both by electron and by conventional light microscopy.

Indirect immunofluorescence studies revealed that the antibodies elicited by the histones purified from calf thymus cross react with the salamander lampbrush chromosomes. Microinjection of the anti-histone antibodies resulted in retraction of the transcription loops. This was observable both by light and by electron microscopy. In control experiments, identical amounts of IgG molecules specific to a homologous RNA-binding protein were microinjected. In such controls, no morphological alteration of the lampbrush chromosome could be observed. Thus, H2B antibodies react specifically with histone associated with transcribed DNA in their native state. The resulting antigen-antibody complex inhibits progression of the RNA polymerases along the template, thereby causing premature release of transcripts. The demonstration

of histones associated with heavily transcribed regions which are not compacted into nucleosomes suggests that the unfolding of the chromatin subunit during transcription does not involve dissociation of histones.

This is the first report of microinjection of specific antibodies to study the function of chromosomal proteins. The possibility of using mammalian cells for the same purpose is currently examined.

Significance to Biomedical Research and the Program of the Institute: Understanding the mechanism of gene regulation and its relation to neoplasia requires knowledge of the structure of chromatin and chromosomes. The approach developed in this laboratory is presently the only approach in which specific probes for well-defined purified chromosomal components are used to study the organization of these components in intact chromatin and chromosomes. As such, a unique opportunity has developed whereby certain structural aspects of these nucleoproteins can be visualized and directly related to functional stages of the genome. The immunological techniques developed for the study of the in situ organization of proteins in chromatin and chromosomes are applicable to studies on damage and repair in the genome as a result of binding of carcinogens or X-ray and UV exposure.

Proposed Course of Project: Studies devoted to understanding the structure-function relation of defined chromosomal components will be continued. The main methodological approach will be serological since significant experience with these approaches has been obtained. The efforts will be concentrated on 1) elucidation of the location of certain chromosomal proteins in the various regions of polytene chromosomes, 2) exploring the feasibility of microinjection of functional proteins into cells and 3) using serological techniques to purify nucleosomes which contain selected antigens.

Publications

Bustin, M.: Immunological approaches to chromatin and chromosome structure and function. Current Topics in Microbiol. Immunol. 88: 105, 1979.

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Romani, M., Vidali, G., Tahourdin, C. S. M., and Bustin, M.: Solid-phase radioimmunoassay for chromosomal components. J. Biol. Chem. 255: 468, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04499-03 LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Separation, Identification and Quantitation of Cytochromes P-450 in Mammalian Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cytochrome P-450 is a major component of a mixed-function oxidase enzyme system which catalyzes the metabolism of many compounds, including polycyclic hydrocarbons known to produce cancer in humans. Several forms of cytochrome P-450 differing in activity and substrate specificity are known to exist in mammalian liver. The ability of the individual to activate or de-activate chemical carcinogens and thus his susceptibility to cancer induction by a given agent may depend on the absolute amounts or relative proportions of particular cytochrome P-450 species present. Hence, it would be useful to be able to recognize and quantitate the cytochrome P-450 forms. In this study, methods for the identification of these proteins have been developed using rat and rabbit liver microsomes and currently are being used to analyze cytochromes P-450 of human monocytes. The methods employed effectively combine isoelectric focusing, column chromatography, enzymatic assay, SDS-polyacrylamide gel electrophoresis and spectrophotometry and should be useful for examining cytochrome P-450 content of low activity as well as high activity tissues.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of this study are: 1) To detect and quantitate multiple cytochrome P-450 forms in various animal and human tissues and to determine the effects of exposure to carcinogenic polycyclic hydrocarbons (e.g., 3-methylcholanthrene and benzo(a)pyrene) on the amounts and proportions of these cytochromes present, 2) To determine whether there is a relationship between type and/or amount of cytochrome P-450 present in a tissue and the incidence of cancer arising upon exposure to a given carcinogen, and 3) To clarify the nature of induction of cytochrome P-450 and mixed-function oxidase activity by polycyclic hydrocarbons.

Methods Employed: An electrofocusing method has been developed to discriminate between different forms of cytochrome P-450. Microsomes or tissue samples are solubilized using a non-denaturing detergent and the resulting solution is subjected to isoelectric focusing either in polyacrylamide gel or in a liquid column containing a glycerol gradient. Slices of the focused gel or aliquots of the focused column are assayed for a cytochrome P-450-dependent reaction, i.e., benzo(a)pyrene (BP) hydroxylation. Recovery of activity from polyacrylamide gels is variable, but the discrimination between two forms of cytochrome P-450; namely, the major uninduced rat liver cytochrome P-450 and the 3-methylcholanthrene (MC)-induced rat liver cytochrome P-450, is sufficient to distinguish these forms in tissues having fairly high benzo(a)pyrene hydroxylase activity, such as liver microsomes. For tissues having lower activities, such as human monocytes, more promising results have been obtained by focusing the solubilized preparation on a liquid column. Recovery of activity of these samples is generally higher when the focusing is done using the liquid column than when a polyacrylamide gel is used. Also, anion exchange column chromatography has proven useful for partially purifying monocyte cytochrome P-450 prior to analysis by electrofocusing or other methods.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing of purified cytochromes P-450 followed by staining of the gels for protein also have proven useful in analyzing the banding patterns and identifying cytochrome P-450 bands of microsomal proteins similarly electrophoresed. Spectrophotometric quantitation of the resolved cytochrome(s) P-450 after elution from the gels is being attempted and immunological reactivity of these proteins with antibody to pure cytochrome(s) P-450 may be examined.

For the development of the analytical methods, rat and rabbit liver have been used as the source of the cytochrome P-450. Human blood monocytes treated with benz(a)anthracene in vitro to raise the cytochrome P-450 and mixed-function oxidase levels have been the major sample examined in the initial application of these analytical methods to human tissue. Subsequent analysis of uninduced human monocytes and uninduced and benz(a)anthracene (BA)-induced human lymphocytes also will be undertaken.

Major Findings: Analysis of protein banding patterns after SDS-PAGE or isoelectric focusing of liver microsomes from uninduced and 3-methylcholanthrene (3-MC)-induced rats reveals clear differences between the two samples. Follow-

ing SDS-PAGE, 3-MC-induced microsomes show a prominent band of about 54,000 daltons which is largely absent in the uninduced microsomes. In addition, when these samples are subjected to isoelectric focusing, the apparent isoelectric point (pI) of the mixed-function oxidase activity of the 3-MC-induced microsomes is found to be about 4.7 while that of the mixed-function oxidase activity of uninduced microsomes is about 5.3. In the case of human monocytes and lymphocytes and some mammalian cells in tissue culture where the cytochrome P-450 levels and enzymatic activity are much lower than in liver microsomes, no differences in the SDS-PAGE protein banding patterns are seen though benzo(a)pyrene hydroxylase activity is elevated markedly. Upon isoelectric focusing, the benzo(a)pyrene hydroxylase activity of BA-induced human monocytes and BA-induced BRL cells focused into a single broad band having a pI of 4.3-5.5.

Significance to Biomedical Research and the Program of the Institute: This study should clarify the relationship between drug metabolizing ability, cytochrome P-450 content and type and chemical carcinogenesis, particularly that caused by polycyclic hydrocarbons.

Proposed Course of Project: During this research period the cytochrome P-450 composition of human monocytes and possibly other tissues such as placenta will be examined more thoroughly using the analytical methods which have been developed in this study.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04516-04 LMC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cellular and Molecular Effects of Psoralen Plus Ultraviolet Light

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:		OTHER:
2.3	1.1	1.2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Psoralen plus longwavelength ultraviolet radiation(UV-A) is being investigated as a model system for clinically relevant photochemical carcinogenesis. Used experimentally for treatment of psoriasis and mycosis fungoides, psoralen plus UV-A has been found to be mutagenic in bacteria and carcinogenic in mice and humans. We have developed an in vitro assay to measure the effects of psoralen-DNA binding on human lymphoid cells. Parameters monitored include the rate of DNA synthesis, induction of DNA-psoralen cross-links, induced sister chromatid exchanges, alterations in the rate of cell proliferation and survival, and in immune reactivity. We are attempting to measure psoralen-DNA antibodies in rabbits or humans for use as a cellular probe. These studies are aimed at understanding the mechanism of cell damage induced by psoralen plus UV-A so as to minimize the toxicity to human cells during therapy.

Project Description

Humans are exposed to chemicals in the environment and as medical therapy which may interact with ultraviolet radiation to become carcinogenic. 8-methoxypsoralen (8-MOP), a compound which is found in many plants, plus high intensity long-wavelength ultraviolet radiation (UV-A) is being used experimentally to induce remissions in psoriasis and in mycosis fungoides. The combination of 8-MOP plus UV-A produces DNA-8-MOP binding and has been shown to induce mutations in bacteria and in mammalian cells and to cause skin cancer in mice and humans.

We previously demonstrated that circulating lymphoid cells of some psoriasis patients receiving 8-MOP plus UV-A therapy had a significant reduction in DNA synthesis. We have developed an in vitro assay system to approximate some of the conditions of 8-MOP plus UV-A on human lymphoid cell in vivo during therapy. The assay has been used with fresh lymphocytes and with long term lymphoblastoid cell lines. These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells.

We have developed a simple microtiter assay to measure lymphoblastoid cell survival after treatment with 8-MOP plus UV-A or other DNA damaging agents. We are currently attempting to automate the end point analysis of this assay.

In the lymphoblastoid cells, as much as a 50% inhibition of DNA synthesis following 8-MOP plus UV-A treatment was associated with 100% survival. Greater inhibition of DNA synthesis resulted in an exponential decrease in cell survival. Similarly, measurements of 8-MOP-DNA cross-linking by the alkaline elution technique revealed a dose dependent increase in cross-link induction above a threshold of approximately 50% inhibition of DNA synthesis. The formation of cross-links was also correlated with decreased cell survival. Thus DNA-8-MOP interstrand cross-links may be responsible for inhibition of DNA synthesis and cell killing.

8-MOP plus UV-A treatment of lymphocytes of lymphoblastoid cells in vitro resulted in approximately a doubling in the number of sister chromatid exchanges per metaphase. Further increases in 8-MOP plus UV-A were toxic. Thus it is likely that the doses of 8-MOP plus UV-A received by patients' lymphocytes are too low to permit routine detection of increased sister chromatid exchanges.

Mixed leukocyte reactivity of fresh human leukocytes was found to be inhibited a dose dependent manner by 8-MOP plus UV-A in vitro. This inhibition of immune reactivity may be related to in vivo carcinogenesis.

An immunoassay is being developed to measure 8-MOP-DNA adducts. We are attempting to produce these antibodies in immunized rabbits. Concurrently, we are examining sera of 8-MOP plus UV-A treated psorasis patients for

evidence of 8-MOP-DNA antibodies. We plant to a) attempt to correlate the presence of these antibodies with clinical symptoms and b) use the antibodies to monitor formation and removal (repair) of 8-MOP-DNA adducts in vitro and in vivo.

Cancer patients who develop cutaneous infection with varicella-zoster virus may suffer increased morbidity and mortality. 8-MOP plus UV-A has been demonstrated to decrease herpes virus multiplication in mice. In collaboration with investigators in the Pediatric Oncology Branch we are studying the therapeutic efficacy of 8-MOP plus UV-A for cancer patients with herpes zoster infection. This laboratory provides dermatologic expertize and phototherapy equipment. Patients have recently begun treatment under a controlled protocol.

Publications

Kraemer, K.H., Waters, H.L., Ellingson, O.L., and Tarone, R.E.: Psoralen plus ultraviolet radiation-induced inhibition of DNA synthesis and viability in human lymphoid cells in vitro. Photochem. Photobiol. 30: 263-270, 1979.

Kraemer, K.H., Waters, H.L., and Buchanan, J.K.: Survival of human lymphoblastoid cells after DNA damage measured by growth in microtiter wells. Mutat Res (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CP 04517-04 LMC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

DNA Repair In Human Cancer-Prone Genetic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.H. Kraemer	Research Scientist	LMC	NCI
OTHER:	W.L. Levis	Senior Investigator	D	NCI
	D. Scudiero	Senior Staff Fellow	LMC	NCI
	L.F. Cohen	Investigator	PO	NCI
	P.H. Kohn	Cancer Expert	M	NCI
	S.F. Stinson	Pathologist	TPB	NCI
	M.H. Greene	Clinical Epidemiologist	EB	NCI
	H.V. Gelboin	Chief	LMC	NCI
	J. Bader	Clinical Investigator	CEB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human cancer-prone genetic diseases are being studied in order to identify groups of people with an increased susceptability to environmental carcinogenesis, and to attempt to correlate such sensitivity with clinical features and with defective DNA repair. Patients with xeroderma pigmentosum and ataxia telangiectasia, diseases with ultraviolet and X-ray sensitivity, respectively, and with familial malignant melanoma (dysplastic nevus syndrome), and neurofibromatosis, diseases with no documented environmental sensitivity, are being studied. Detailed examinations of the clinical features of affected individuals are being made. Cultures of skin and blood are being established and the effects on cell survival, DNA repair, and chromosome abnormalities after treatment with DNA damaging agents (ultraviolet, bleomycin, and psoralen plus long wavelength ultraviolet) are being examined.

PHS-6040
(Rev. 10-76)

Project No. Z01-CP-04517-04-LMC

PROJECT DESCRIPTION

Human cancer-prone genetic diseases are being studied with a view toward identifying groups of people with an increased susceptability to environmental carcinogenesis, to determine if there is genetic diversity within such groups, and to attempt to correlate such sensitivity with clinical features and with defective DNA repair. Patients are examined with particular emphasis on cutaneous abnormalities and cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory experimentation. Patients with xeroderma pigmentosum (XP), ataxia telangiectasia (AT), familial malignant malanoma (dysplastic nevus syndrome, DNS) and neurofibromatosis (NF) have been examined.

XP, an autosomal recessive cancer prone disease with clinical ultraviolet (UV) sensitivity, and cutaneous and neurological abnormalities, also has cellular UV sensitivity and defective DNA repair. We have compiled a comprehensive review of the world literature in XP including clinical and experimental observations. We have developed a semi-automated technique to facilitate the autoradiographic measurement of DNA repair in XP cells. Selected XP patients with unusual clinical features are being studied for cellular UV sensitivity and abnormal DNA repair.

AT, an autosomal recessive cancer-prone disease with cutaneous, neurological and immunological abnormalities has X-ray sensitivity. We are studying the ability of cultured cells from AT patients to survive DNA damage induced by the chemotherapeutic agent, bleomycin. We have found that AT lymphoblastoid cell lines are abnormally sensitive to killing by bleomycin and that this agent induces an abnormally large increase in chromosome breakage (but not in sister chromatid exchanges) in AT lymphoblastoid cells. Analysis of repair of bleomycin-induced DNA damage by use of the alkaline elution assay is under way. Studies of the specificity of the AT cellular defect are in progress using UV and psoralen as other DNA damaging agents. We are also attempting to determine if cells from heterozygous carriers of AT have abnormal bleomycin response.

A newly recognized clinical disease, familial malignant melanoma with a characteristic precursor legion, the dysplastic nevus, is being examined in collaboration with the Environmental Epidemiology Branch, NCI. This laboratory is contributing dermatological expertize in the clinical definition of the syndrome in a study of more than 300 family members. Lymphoblastoid cell lines from selected patients are being examined for evidence of sensitivity to DNA agents, and possible DNA repair defects.

NF, an autosomal dominant disease with characteristic cutaneous pigmentary lesions and multiple nervous system tumors is being examined in collaboration with the Clinical Epidemiology Branch, NCI. Selected patients with different forms of NF are examined clinically and cultures of skin and blood are established. This laboratory is providing dermatological advice and expertize in examination of the patients and will study cultured cells for evidence of sensitivity to DNA damaging agents.

Patients with psoriasis, a benign disease with a strong genetic component, have rapid, but non-neoplastic proliforation of lesional epidermal cells. We are studying the level of aryhydrocarbon hydroxylase (AHH), an important enzyme in carcinogen metabolism, with lymphocytes and monocytes from psoriasis patients in collaboration with the Metabolic Control Section, LMC. This laboratory is examining the patients clinically and obtaining blood for analysis. Preliminary observations indicate age related changes in AHH levels in monocytes and lymphocytes and increased AHH levels specifically in lymphocytes from patients with psoriasis.

Publications

Kraemer, K.H., Buchanan, J.K., and Stinson, S.F.: Semi-Automated autoradiographic grain counting instruments for measurement of DNA repair. In Vitro (In Press).

Kraemer, K.H.: Xeroderma Pigmentosum: A prototype disease of environmental-genetic interaction. Arch. Dermatol. (In Press).

Kraemer, K.H.: Xeroderma Pigmentosum. In Demis, D.J., Dobson, R.L. and McGuire, J. (Eds): Clinical Dermatology. New York, Harper and Row Publishers (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04518-04 LMC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

AA-tRNAs in Protein Synthesis and Evolutionary Changes in AA-tRNA and Synthetase Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Hatfield	Research Biologist	LMC NCI
OTHER:	M. Rice	Chemist	LMC NCI
	E. Hoffman	Biological Aide	LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Relative utilization of isoacceptor aa-tRNAs in protein synthesis have demonstrated variations in the incorporation of the corresponding amino acid into protein even though the isoacceptors have the same codon recognition properties. Two minor Ser-tRNAs which recognize specifically the nonsense codon UGA have been shown to be conserved in higher vertebrates. Patterns in codon recognition of isoacceptor aa-tRNAs from Physarum polycephalum have been determined and the aminoacylation of 19 aa-tRNAs fractionated from several species under homologous and heterologous conditions have been examined.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives were: 1) To determine the effect of specific base modifications in isoacceptor aa-tRNAs on protein synthesis and whether misreading genetic code words by isoacceptor aa-tRNAs during protein synthesis may be involved in the expression of cancer; 2) To determine the cellular function of minor Ser-tRNAs which recognize the nonsense codon UGA ($Ser\text{-}tRNA_{UGA}$); and 3) To examine evolutionary changes in the aminoacylation of tRNA and in anticodon: codon interactions in a variety of species in order to elucidate the means by which genetic information which is carried in one region of a genome (e.g., tRNA gene) and is coded to interact with genetic information carried in another region (e.g., in an aa-tRNA gene) undergoes evolutionary change.

Methods Employed: Labeled aa-tRNAs were added to rabbit reticulocyte lysates and to wheat germ extracts and the relative rates of incorporation of the corresponding amino acid from each isoacceptor into protein determined by chromotography of the aa-tRNA remaining after protein synthesis. Minor Ser-tRNAs which recognize specifically the nonsense codon UGA have been isolated from bovine liver by a simple procedure which permits the processing of large quantities of tissue. This procedure entails making an extract of bovine liver followed by the attachment of the more hydrophobic tRNAs to DEAE-cellulose, subsequent removal of $tRNA^{Ser}$ from DEAE cellulose and attachment to and removal from BD-cellulose.

Eleven aa-tRNAs from Physarum have been fractionated by reverse phase chromatography and the codon recognition properies of the individual isoacceptors determined by a ribosomal binding assay. Transfer RNA from E. coli, yeast and wheat germ have been fractionated by reverse phase chromatography and the ability of aa-tRNA synthetases from E. coli, yeast, wheat germ and rabbit reticulocytes to aminoacylate 19 tRNAs under homologous and heterologous conditions determined by standard aminoacylation assays.

Major Findings: The technique which determined the relative utilization of isoacceptor aa-tRNAs in protein synthesis and was previously established in this laboratory has unequivocally demonstrated that an isoacceptor of Lys-tRNA, which has been shown in other laboratories to be correlated with cellular division, was not utilized as rapidly as other isoacceptors with the same codon recognition properties in wheat germ extracts programmed with mRNA. If this isoacceptor manifests similar affinity for the elongation factors as other aa-tRNAs, then it could play a role in controlling the rate of protein synthesis.

We have previously shown that Phe-tRNA which contains Wye base and Phe-tRNA which lacks Wye base (from FrNTD and neuroblastoma cells) transfer phenylalanine to protein at similar, but not identical rates. This past year, the rates of incorporation of phenylalanine from highly purified, double labeled isoacceptors into globin in rabbit reticulocyte lysates have been carefully examined. Although neuroblastoma Phe-tRNA appears to donate its phenylalanine

to protein more rapidly than normal Phe-tRNA, this observation must be confirmed in wheat germ extracts programmed with globin mRNA. (Studies on Phe-tRNA with and without Wye base are a collaboration with Dr. J. F. Mushinski, LCBGY, NCI).

Minor Ser-tRNAs which recognize UGA have been shown to occur in Xenopus liver. These studies along with previous studies in this laboratory which have shown the occurrence of these two isoacceptors in several mammalian tissues and in chicken liver demonstrate that these isoacceptors have been conserved in higher vertebrates. The serine attached to one of the two isoacceptors in phosphorolated on Ser-tRNA (A. Diamond and B. Dudock, personal communication). The cellular role of both isoacceptors is presently being examined in this laboratory.

Patterns of codon recognition of isoacceptor aa-tRNAs fractionated from Physarum aa-tRNAs have been determined. It is of interest to note that the patterns of codon recognition of Physarum aa-tRNAs are more similar to those previously observed in the animal kingdom (see Hatfield, Rice and Hoffman, Biochem. Biophys. Res. Comm. 85: 436, 1978 and Hatfield, Mattthews and Rice, Biochim. Biophys. Acta 564: 414, 1979, for patterns of codon recognition of isoacceptor aa-tRNAs from hymenoptera and from mammals, respectively) than those observed in the plant kingdom (see Hatfield and Rice, Nucleic Acids Res. 5: 3491, 1978, for patterns of codon recognition of isoacceptor aa-tRNAs from wheat germ). (Studies on Physarum aa-tRNAs are a collaboration with Dr. Peter Melera of the Sloan Kettering Institute.)

Cross aminoacylation studies with tRNAs fractionated from E. coli, yeast and wheat germ with synthetases from these organisms and, in addition, rabbit reticulocytes have extended earlier studies in this laboratory which were carried out with bulk tRNA. Several cases where complete aminoacylation of one isoacceptor and little or no aminoacylation of a second isoacceptor were observed under heterologous conditions. Such evolutionary changes provide evidence that synthetases recognize their cognate isoacceptors in different ways. No examples of efficient misrecognition were observed. These studies complete our investigations on evolutionary changes in tRNA synthetase and AA-tRNA(anticodon):codon interacitons.

Significance to Biomdical Research and the Program of the Institute: Major unresolved questions in biology are whether tRNA may play a role in cellular regulation and in carcinogenesis. Approaches to understanding these problems are to determine if specific isoacceptor aa-tRNAs can influence the rate of protein synthesis and if misreading of isoacceptor aa-tRNAs in protein synthesis can induce cell transformation.

Proposed Course of Project: The proposed course is: 1) to determine if the isoacceptor of Lys-tRNA which is utilized less frequently in protein synthesis has a similar affinity for the elongation factors as other aa-tRNAs; 2) To determine if Phe-tRNA minus Wye base transfers its phenylalanine more

rapidly to protein than normal Phe-tRNA and, if this observation is substantiated, then to determine if Phe-tRNA minus Wye base may transfer its amino acid into protein sites not coded by phenylalanine; and 3) To determine the cellular role of Ser-$tRNA_{UGA}$.

Publications

Hatfield, D., Matthews, C. R. and Rice, M.: AA-tRNA populations in mammalian cells. Part I. Chromatographic profiles and patterns of codon recognition. Biochim. Biophys. Acta 564: 414, 1979.

Hatfield, D., Richer, L., Lyon, J. and Rice, M.: AA-tRNA populations in mammalian cells. Part II. Relative utilization of mammalian Lys-tRNA isoacceptors in protein synthesis. FEBS Letters, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04525-08 LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on Electrophoretic Techniques for Protein, RNA, and DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. C. Peacock Chief, Protein Section LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	0.8	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new method for estimation of chain lengths of ribonucleic acids is under development. The method involves partial heat degradation of the RNA under study and measurements of the remaining and degraded molecules. From similar measurements on reference RNAs the chain lengths may be deduced. In addition, computer simulation permits computation of the relation between chain length and electrophoretic mobility for use in electrophoretic analysis of polydisperse samples. Heterogeneous nuclear RNA from HeLa cells has been studied by these techniques. Purification of RNA and DNA by means of conventional and electrophoretic techniques has been achieved, principally through the use of apparatus designed elsewhere. The purified material facilitates study of the properties of these molecules.

PHS-6040
(Rev. 10-76)

. Project Description

Objectives: To investigate the influence of primary structure and conformation on the electrophoretic and biological properties of proteins and nucleic acids and to use this information in devising improved methodology for analysis and purification. To study the role of hnRNA in cellular control in normal and malignant cells by these techniques. To develop suitable methodology for the preparation of hnRNA in undegraded and native form.

Methods Employed: Cultures of HeLa cells, assay of radioisotopes, isolation and electrophoresis of RNA and DNA, optical scanning of stained and unstained gels, preparative electrophoresis, optical measurement of melting of nucleic acids, ultracentrifugal analysis of RNA and DNA.

Major Findings: Considerable effort has been devoted to the production of computer programs which are both easy to use and proceed directly to solutions with minimal use of computer time. At the start of this reporting period the programs which had been written provided reliable estimates of hnRNA size distributions, but were tedious to use and depended to some extent upon the user's prior experience and knowledge of previous solutions. Because the mathematical solution constituted the major time-consuming phase of the analysis, it was felt important to simplify this process as much as possible. Considerable progress has been made toward this end.

In order to characterize hnRNA preparations, they must be free of DNA. The removal of DNA by the use of DNase has been found to be the only practical approach. Unfortunately, commercially available preparations of DNase unavoidably contain small amounts of ribonuclease. Attempts were made to purify DNase by several different published methods and to estimate the ribonuclease content before and after purification. At least one published method has given good results, but the variable content of ribonuclease initially present requires careful monitoring of this reagent. Improved electrophoretic methods have been developed which use purified nucleic acids to assess the quality of the DNase used in the preparation of hnRNA. In addition, several different approaches to the preparation of hnRNA that might reveal details concerning its native conformation are being investigated.

Significance to Biomedical Research and the Program of the Institute: The ability to estimate chain lengths of polydisperse ribonucleic acids by methods dependent on molecular properties very different from those previously available (centrifuge, electron microscope, etc.) provides a check on these methods. It also permits modifications in sample preparation, a step which may introduce ambiguities.

The heterogeneous RNA is the primary transcription of the gene and, in addition to specific messengers for specific proteins, presumably contains all RNA that might be involved in the control of genetic expression. Analytical methods for studying this RNA and assessing its role may be fundamental to understanding the alterations which occur in malignancy.

Proposed Course of Project: 1) The properties of functional mRNA and the original unspliced hnRNA will be contrasted and compared with rRNA to provide insight into the possible structural differences that may characterize these molecules; 2) The ability of hnRNA to self-associate and also to hybridize with DNA will be explored, especially as these may reflect characteristic properties of the transformed state.

Publications

McIntosh, P. R., Peacock, A. C., and Gelboin, H. V.: The induction of liver microsomal proteins by 3-methylcholanthrene in rats of different age, sex and strain. Biochim. Biophys. Acta 627: 290-300, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04554-06 LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Development of the System for Assay of In Vitro Chemical Carcinogenesis and Mutagenesis Using Human and Mouse Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakunaga	Chief, Cell Genetics Section	LMC	NCI
OTHER:	T. Hirakawa	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to study the mechanism of cell transformation, to obtain information about how to extrapolate the results obtained from the carcinogenesis experiments in animals into humans, and to compare the mutation and transformation, it is useful to have a reliable, rapid and quantitative system for simultaneous assays of malignant transformation and mutation in the cells of different species and origins including human cells. The system for malignant transformation of human diploid cells so far developed is not yet rapid and quantitative and the system to compare the induction of transformation and mutation needs to be modified. The purpose of this work is to improve the system for simultaneous assay of induction of mutation and transformation by chemical and physical carcinogens in human diploid fibroblasts and mouse cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To establish an optimum system for simultaneous and quantitative assay of in vitro chemical induced transformation and mutation of human and mouse cells.

Methods Employed: Standard tissue culture techniques of our laboratory. Transformation assays previously developed by us (Int. J. Cancer 12, 463, 1973 and Proc. Natl. Acad. Sci., U.S.A. 75, 1334, 1978). Mutation assays measuring conversion of ouabain-sensitive cells to ouabain-resistant cells.

Major Findings: 1) There had been no established procedures of transformation assays which include replating after carcinogen treatment. A replating step is required for mutation assay in order to avoid metabolic cooperation effects. Under an optimal condition of cell density and time of replating, the transformation frequency induced by chemical carcinogens in A31-1-1 cells reached a similar level to that without replating procedures, thus making it possible to assay the mutation and transformation frequency under the same condition of expression as well as the condition of exposure to carcinogens; 2) Because different doses of chemicals or UV caused different levels of delay of cell generation, and because there was an optimum expression time beyond which there were marked decreases in mutation frequency, expression time required for maximum mutation frequency varied with the dose of carcinogens. The number of cell generations after carcinogen treatment rather than the time allowed for expression was crucial to obtain the maximum mutation frequency; 3) The examination of the selective condition for the transformed human fibroblast cells against untransformed cells has revealed that the ability of the cells to grow over the monolayer sheet of untransformed cells, that resulted in focus formation, was the clearest and most objective indicator of the transformation of human fibroblasts and that this property could be used as an efficient condition of selective growth of the transformed cells. Culture in soft agar gave an advantage for growth of transformed cells but not marked. Low concentration of calcium ion and/or serum in culture medium that are known to be the selective growth conditions for many transformed cells as opposed to untransformed cells did not give significant advantage of growth and survival to the chemically transformed human fibroblasts compared to the parental untransformed cells.

Significance to Biomedical Research and the Program of the Institute: This project will provide a system which is useful to study the mechanism of cell transformation and mutation and their relation. It would also provide information on differences in the response to the carcinogens between different species, and between different cell systems such as freshly isolated cells vs. established-line and in vitro vs. in vivo. The information thus obtained would be very helpful for extrapolating the results of animal experiments or in vitro systems into assessment of human risk against environmental carcinogens.

Publications

Kakunaga, T., Crow, J. D., and Augl, C.: Comparative studies on the induction of mutation and malignant transformation in mouse and human cells. In Okada, S., Imamura, M., Terashima, T. and Yamaguchi, H. (Eds.): Radiation Research. Tokyo, Japan, Assoc. Rad. Res., 1979, pp. 589-595.

Kakunaga, T.: Transformation system of subclones of Balb/3T3 cell line. Tissue Culture 5: 405-412, 1979.

Kakunaga, T.: Approaches towards the development of human transformation assay system. In Mishra, N. K., Dunkel, V. and Mehlman, M. A. (Eds.): Advances in Environmental Toxicology; Mammalian Cell Transformation by Chemical Carcinogens. New York, Pathotex Publishers, In press.

Kakunaga, T.: Assay of chemically-induced transformation of human cells. In: Stich, H. F. and San, R. H. C. (Eds.): Short Term Tests for Chemical Carcinogens. New York, Springer-Verlag, In press.

Kakunaga, T: In vitro chemical carcinogenesis of human cells. Metabolism, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04555-06 LMC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Factors Affecting Malignant Transformation and Mutation by Chemical Carcinogens in Human and Mouse Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakunaga	Chief, Cell Genetics Section	LMC NCI
OTHER:	T. Hirakawa	Visiting Fellow	LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to clarify the mechanism of cell transformation by examining the genetic and physiological factors which affect cell transformation induced by chemicals and irradiation. Comparative studies of the susceptibilities to transformation and mutation induction between cell variants and the effects of growth condition after ultraviolet (UV) radiation on the fixation and expression of transformation and mutation induction suggest the existence of a difference in the process of induction between transformation and mutation although there are similarities between the two processes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To know about the process of malignant cell transformation induced by chemical and physical carcinogens by defining and identifying the genetic and physiological factors affecting the transformation.

Current Interests are: 1) To clarify the cause of different susceptibility of cell variants to the chemical- and UV-induced cell transfromation. 2) To compare the effect of post-UV cell growth condition on the induction of transformation and mutation.

Methods Employed: A quantitative system for assay of ouabain-resistance mutation (dominant) and malignant tranfromation induced by UV in subclones of A31-1 cells derived from Balb/3T3 mouse cell line was used that has been developed in our laboratory (see project No. Z01-CP-04554).

Major Findings: 1) Cell variants showing different susceptibility to the transformation induced by chemical carcinogens or UV which have been isolated in our laboratory showed similar sensitivity to the induction of ouabain resistance mutation by UV at any dose tested. 2) Optimum expression time for mutation varied with the dose of UV. However, the number of cell generations required for the maximum expression of mutation was constantly about two. Our previous results indicated that at least 4 cell genrations were required for expression of transformation. 3) The cells maintained at confluent and nongrowing state after UV-radiation could not express the mutated phenotype as well as the transformed phenotype. 4) When the cells held at confluent and nongrowing state after UV-radiation were replated sparsely so that they could divide thereafter, the observed frequency of UV-induced mutation, regardless of length of time held at nongrowing conditions, was similar to the frequency found in cells which were maintained at growing state all the time after UV-radiation. On the other hand, transformation frequency decreased gradually with the time of holding the cells at nongrowing condition after UV-radiation. The decrease rate was slower than that of A31-714 cells which lost the ability to be transformed very rapidly and the ability to be mutated at a mild rate under the same conditions as have been previously reported by us. These results indicate that about 2 or 4 cell generations are necessary for expression of dominant mutation or transformation, respectively, and that there are differences in the rate of loss of fixation while holding the cells at nongrowing conditions after UV-radiation between mutation and transformation induced by UV in subclone of A31-1 cells and between A31-714 and A31-1-1 cells.

Significance to Biomedical Research and the Program of the Institute: This project will provide: 1) Information on the development and improvement of the system for assay of chemical and physical carcinogens and mutagens. 2) Information about the mechansim of cell transformation

by carcinogens. 3). Basic information about the environmental, genetic, and physiological factors in the incidence of human cancer. Thus, this project directly aims at the aspect of the Proram of the Institue which seeks to find rapid meaningul assays for environmental carcinogens and ways to prevent cancer incidences.

Proposed Course of Project: 1) To continue to investigate the cause of the different susceptibility to transformation among subclones of A31-I cells. 2) To compare the excison repair activity between A31-I-1 cells and A31-714 cells and study the relation of DNA damage induced by carcinogens, its repair, and induction of mutation and transformation. It has been shown that the A31-714 cells lost the ability to be transformed and mutated rapidly while being held at nongrowing condition after carcinogen treatment and excised rapidly the 4-nitroquinoline-1-oxide-DNA adducts (see Z01-CP-04555 in the past years). In contrast, the A31-1-1 cells lost little or none of the ability to be transformed or mutated under similar conditions (as reported here) and showed poor ability to excise benzo [a]pyrene-DNA adducts (see Z01-CP-5001-03).
3) To compare the susceptibility to UV- or chemical-induced transformation between the cells obtained from normal humans and cancer-prone gentic disease patients. There has been no clear experimental evidence that the cells from cancer-prone genetic disease patients have a higher susceptibility to the transformation induced by chemicals or irradiation at cellular level than the cells from normal humans.
4) To pursue the goals outlined in Objectives and to publish the results so obtained.

PUBLICATIONS:

Kakunaga, T. and Crow, J.D.: Cell variants of different sensitivity to ultraviolet light-induced cell transformation. Science, (In Press).

Kakunaga, T.: Chemically-induced malignant transformatin of mammalian cells. In Kuroki, T., Okada, Y. and Yamane, S. (Eds): Somatic Cell Genetics. Tokyo, Rikogakusha, (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 04578-04 LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Genes Responsible for the Transformed Phenotype in Chemically Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakunaga	Chief, Cell Genetics Section	LMC NCI
	H. Hamada	Visiting Associate	LMC NCI
	T. Hirakawa	Visiting Fellow	LMC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this work to clarify the nature of cellular molecules responsible for the malignant phenotype of chemically transformed cells. Although many attempts have failed to identify such a molecule, recent progress in molecular biology and somatic cell genetics have provided new methods to isolate and characterize the cellular genes and their products which seem very useful for this objective. Two approaches are taken. One approach is an attempt to isolate cellular genes which directly control the expression of transformed phenotype and another approach is to clarify the structure and expression mechanism of the genes coding macromolecules, alterations of which have been suggested to be associated with the expression of transformed phenotype. Preliminary results indicate the possibility to isolate genes controlling the transformed phenotype of human cells by using transfection and gene cloning techniques. Actin, a major protein in the cytoskeleton, has been considered to be involved in many cellular functions including expression of transformed phenotype. An altered form of actin molecule was found in the chemically transformed human fibroblast cells. cDNA synthesized from actin mRNA of these human cells was inserted into plasmid pBR322 DNA.

Project Description

Objectives: To learn about genetic regulation of the expression of transformed phenotype and to know the biochemical nature of the macromolecules controlling the expression of the transformed phenotype. Current Interests are: 1) To know whether the transformed phenotype is expressed in a complete hybrid cell between a normal human diploid fibroblast cell and its chemical-transformant. 2) To establish a system for transfection in human diploid fibroblasts using biological and biochemical markers. 3) To know whether DNA extracted from chemically transformed human cells will transfer the malignant phenotype into normal diploid human cells. 4) To isolate plasmid containing cDNA synthesized from mRNA specific for the normal and mutated form of actin (A'-actin) which was found in chemically transformed human cells and to clone genomic actin and A'-actin gene DNA sequence.

Methods Employed: Standard tissue culture techniques of our laboratory, karyotype analysis using chromosome banding techniques, transplantation of the cultured cells into nude mice, DNA transfection, and biochemical techniques such as gel electrophoresis, in vitro translation, amino acid sequencing, ultracentrifugation, DNA-DNA and DNA-RNA hybridization, and other techniques used for gene cloning and recombinant DNA work. Hybrid cells are isolated by selecting and cloning the growing cells in the presence of HAT (hypoxanthin, aminopterine, thimidine) and ouabain after fusing normal human diploid cells (ouabain-sensitive and HAT-resistant) with chemically transformed human cells which are ouabain-resistant and HAT-sensitive.

Major Findings: 1) Careful examination of karyotype by chromosome banding techniques have revealed that among the cells which grew in the selective medium after fusion human diploid fibroblast cells with 4NQO-transformed cells only a small portion of them were 1:1 hybrid cells which retained stably a complete set of chromosomes from both parental cells. These 1:1 hybrid cells showed a transformed phenotype such as morphological alteration, higher saturation density and the high ability to grow over a monolayer of untransformed cells. These preliminary results indicate the dominant expression of in vitro malignant phenotype in 1:1 hybrid cells between normal human fibroblast cells and their 4NQO-transformed counterparts; 2) Exposing normal human diploid cells to DNA extracted from ouabain-resistant human cells resulted in the appearance of ouabain-resistant cells. This is the first demonstration of the successful biological transformation in diploid human cells; 3) When mRNA extracted from 4NQO-transformed human fibroblast cells which has been demonstrated to synthesize an altered form of actin (A'-actin) was translated in vitro using reticulocyte lysate or wheat germ extract, A'-actin was synthesized. This indicates that structural alteration of the actin molecule in the transformed cells is not due to the post-translation modification; 4) cDNA synthesized from actin mRNA of 4NQO-transformed human fibroblast cells has been inserted into pBR322 plasmid DNA.

Significance to Biomedical Research and the Program of the Institute: The results from this project will provide information on the structure and function of the genes and their products controlling the expression of the transformed state, and will also contribute to the understanding of the mechanism of carcinogenesis. Thus, this project is directly aimed at that aspect of the Program of the Institute that seeks to find an essential difference in the biochemical nature between normal and malignant cells and a possible way to present and cure cancer.

Proposed Course of Project: 1) To detect, isolate, and characterize the cellular genes controlling the expression of transformed or normal phenotype of human cells by using transfection and gene cloning techniques; 2) To determine the structure of the genes related to the synthesis of human actin and to know the requlatory mechanism of its gene expression and to know the role of actin in the maintenance of transformed or normal phenotype of human cells; 3) To pursue the goals outlined in Objectives and current interests and to publish the results so obtained.

Publications

Leavitt, J. and Kakunaga, T.: Expression of a variant form of actin and additional polypeptide changes following chemical induced *in vitro* neoplastic transformation of human fibroblasts. *J. Biol. Chem.* 255: 1650-1611, 1980.

Barrett, J. C., Sheela, S., Ohki, K. and Kakunaga, T.: Reexamination of role of plasminogen activator in anchorage-dependent growth of chemically transformed hamster cells. *Cancer Res.*, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04598-05 LMC

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

DNA Repair Deficiency and Increased Probability of Cancer in Certain Human Genetic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. A. Scudiero Senior Staff Fellow LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies comparing the post-treatment behavior of fibroblasts from patients with ataxia telangiectasia with that of fibroblasts from control donors showed then similar behavior after either methyl methane sulfonate (MMS) or ultraviolet (UV) treatment, but different behavior (decreased colony forming ability and usually decreased DNA-repair synthesis) after N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment.

Similar studies using a number of human tumor cell strains showed many of these to be extremely sensitive to MNNG-produced killing, somewhat sensitive to MMS and BCNU (bis-chloroethyl-nitrosourea) killing, but not to UV-produced killing.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To examine the DNA repair capacities of cell strains derived from persons with genetic diseases in which there is a high probability for cancer. Specifically, to measure repair synthesis and cell survival in these cells after treatment with a variety of chemical carcinogens. Also, to examine the effects of inhibitors of semiconservative DNA synthesis on DNA repair and cell survival.

Methods Employed: A simple methodology utilizing benzoylated naphthoylated DEAE cellulose chromatography (BND-cellulose) for the rapid quantitative estimation of excision repair and the rigorous separation of repair synthesis from semiconservative replicative synthesis was employed. In the presence of hydroxyurea, an inhibitor of semiconservative DNA synthesis, the progression of DNA chains is slowed and isotope incorporated by residual replicative synthesis remains in the growing point region. Because the growing point contains single-stranded regions, DNA containing the newly incorporated isotope adheres to the column and requires formamide for elution. Isotope incorporated by repair synthesis into the bulk of the DNA (double-stranded) is eluted with native DNA in the 1.0 M NaCl eluate. After corrections for thymidine pool size are made, the specific repair activity induced by any treatment can be calculated from the radioactivity and absorbency at 260 nm of the 1 M NaCl eluate. By utilizing this method, agents which induce large-patch (UV-like) repair synthesis can easily be distinguished from those inducing small-patch (X-ray-like) repair synthesis. The effects of carcinogen treatment on cell survival was determined by measuring the ability of cells to form colonies. Using colony forming ability in conjunction with the BND-cellulose technique, repair synthesis was correlated with cell survival after carcinogen treatment.

Major Findings:

1) Fibroblasts from six patients with ataxia telangiectasia (AT) were more sensitive than fibroblasts from three control donors to inactivation (colony forming ability) by MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) but not by UV (ultraviolet light) or by MMS (methylmethane sulfonate). The DNA repair synthesis of these AT strains was like that of the control cells after MMS or UV treatment. After MNNG treatment, however, four AT strains showed less DNA repair synthesis than did the control strains or two other AT strains.

2) The effects of novobiocin on DNA repair in cells treated with UV, MMS, or MNNG were studied in collaboration with Dr. Mattern of this section (see report of Dr. Mattern).

3) Studies using MNNG-treated human tumor cells showed a greater than normal amount of DNA repair synthesis in certain os these tumor strains (referred to as Mer$^-$) than in control fibroblast strains or in other tumor strains (Mer$^+$). In terms of colony forming abiliby, Mer$^-$ strains were (by comparison with Mer$^+$ strains) more sensitive to killing by MNNG, MMS, and BCNU (but not by UV). (See report of Dr. Day of this Section for further details.)

4) The Mer$^-$ tumor strains were found (work done with Dr. Day) to lack the ability to excise O^6-methylguanine from their DNA.

Significance to Biomedical Research and the Program of the Institute: Present evidence indicates that chemical carcinogens exert their carcinogenic and mutagenic effects by alterations in cellular DNA. The manner in which cells recognize and eliminate DNA damage is important in assessing an agent's potential for cancer induction. A systematic study of genetic diseases with both a high incidence of cancer and a diminished capacity for DNA repair can lead to useful information concerning the etiology of cancer and the role of DNA repair protecting the organism from potential carcinogenic damage.

Proposed Course of Project: To pursue the goals outlined in Objectives and to publish these results.

Publications

Scudiero, D. A.: Decreased DNA repair synthesis and defective colony-forming of ataxia telangiectasia fibroblast cell strains treated with N-methyl-N'-nitro-N-nitrosoguanidine. Cancer Research 40, 984-990, 1980.

Day, R. S., III, Ziolkowski, C. H. Z., Scudiero, D. A., Meyer, S. A., and Mattern, M. R.: Human tumor cell strains defective in the repair of alkylation damage. Carcinogenesis 1, 21-32, 1980.

Scudiero, Dominic A.: Enhanced sensitivity of N-methyl-N'-nitro-N-nitrosoguanidine of ataxia telangiectasia fibroblasts. In Slor, H, Rickles, E., and Attman, H., (Eds.): DNA Repair and Late Effects. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 - CP - 04782-10 - LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Hormones and Breast Tissue Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. R. Green Research Chemist LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to contribute to the understanding of hormone action on metabolic processes in normal breast tissue under various conditions and to understand the failure of or aberrant response to hormones by hyperplastic or neoplastic breast tissue under the same conditions. Among the topics studied are the response of mammary tissue in culture to hormones in terms of nucleic acid, protein, phosphoprotein, sialoglycoprotein, glycoprotein and glycosaminoglycan synthesis. The kinds of proteins and other macromolecules made by mammary tissue in various stages of differentiation during preneoplasia and by neoplastic mammary tissue are being analyzed. Analyses of rodent and human milks, explants and formalin-fixed rodent and human breast tissue are in progress. Of particular interest are the post-translational modifications of proteins which occur during differentiation and neoplasia.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To gain a clearer understanding of the interaction of hormones with breast tissue. The topics of interest are interaction of hormones such as insulin, steroids, and prolactin with breast tissue components and the metabolic effects of these interactions. Responses by breast tissue to prolactin and steroids in terms of nucleic acid synthesis and synthesis of macromolecules such as phosphoproteins, sialoglycoproteins, glycosaminoglycans and proteins are under investigation as is modulation of these responses in mouse mammary tumor virus containing tissues and in neoplastic tissue. Rodent tissues are examined for preneoplastic changes following carcinogen treatment.

Methods Employed: Culture of mammary tissue explants in vitro using defined medium, isolation and characterization of nucleic acids, separation on agarose-acrylamide gels and on gradients, radioactive tracer techniques, isolation and purification of proteins from milk and explants, measurement of enzymatic activities, SDS-polyacrylamide gel electrophoresis, histochemical analysis of gels and tissue sections, autoradiography, binding of hormones to macromolecules.

Major Findings: Mammary explants from mice in mid-pregnancy incubated in synthetic medium containing insulin, hydrocortisone and prolactin synthesize milk proteins. Molecular weights of four mouse caseins were determined and a κ-like casein of mouse milk was identified. It was shown by densitometric analysis of stained gels that lactoferrin and casein production varied directly in proportion to the amount of prolactin in the medium. Response of explants to prolactin in terms of protein phosphorylation was measured by incubation of explants with ^{3}H-labelled amino acids and ^{33}P-phosphate and by a histochemical method developed previously in this laboratory using the metachromatic dye Ethyl Stains-all. Electrophoretic analysis of proteins synthesized showed that peaks of ^{33}P-phosphorus incorporated into proteins of explants corresponde with the peaks obtained using the histochemical staining method for phosphorus. Several of the phosphorylated proteins of prolactin-stimulated explants corresponded to the caseins of milk. Several additional phosphorylated proteins were detected. These have not been identified. One of these corresponded to a protein found in colostral, but not in mature, milk of the mouse. This protein was characterized further and found to contain carbohydrate and sialic acid.

The histochemical staining procedure was used to determine whether an analogous protein existed in human milk. Human mammary tissue at the lactating state is unavailable for labelling experiments in vitro and the alternative method of labelling proteins in vitro by kinase reactions may label proteins not phosphorylated in vivo. The histochemical method detects by color less than 100 picomoles of phosphorus on gels in 5 μg of casein. Semi-quantitative estimates of the extent of phosphorylation of casein were obtained by mixing various proportions of fully phosphorylated and dephosphorylated caseins while maintaining the same amount of total protein on SDS-PAGE gels. The amount of blue stain of Ethyl Stains-all in the casein band varied directly with the phosphorus content; Coomassie blue staining remained the same.

About fifteen milks from individuals 1 day through 8 1/2 months in lactation were obtained for testing. A phosphoprotein similar in properties to that of the protein of mouse milk was found in most of the human milks examined. It has not been described previously. The reason for this may be that it migrates directly under human serum albumin on SDS gels and it stains weakly with Coomassie blue. Its presence was shown solely on the basis of the histochemical stain for phosphorylated protein. Based on this staining property, this phosphoprotein ranks second in amount to casein in milk. The protein has been isolated and is being characterized further.

Milk proteins from mammary tumor virus containing milks and those not containing virus were separated by SDS-PAGE and compared with respect to milk protein content and staining characteristics for sialic acid and phosphorus. Preliminary experiments showed differences in amounts of these modifiers per amount of protein between these milks. This could be attributed to the presence of the virus. Mammary fat globule membranes of the milk from such mice were similarly examined.

Significance to Biomedical Research and the Program of the Institute: Two phosphorylated glycoproteins not previously described have been isolated from mouse and human milk. The protein of mouse milk, since it is found only in colostrum, may represent a temporarily solubilized microsomal or lysosomal protein. It appears to be present in quantitatively greater amounts than would be expected of an enzyme, however, this has not been excluded. The human protein, while similar to that of the mouse, differs from it by molecular weight, the fact that it is present throughout lactation, and by the type of attachment of sialic acid to its carbohydrate. Knowledge of the constituents of breast tissue and milk is essential for determining changes that occur during the neoplastic process, for identifying breast tissue cells in culture by means of fluorescent antibodies made to specific breast antigens, for developing sensitive radioimmunoassays for recurrent breast disease, and for measuring production of mRNAs for particular proteins and their control by hormones.

Proposed Course of Project: Further chemical and immunochemical characterization of these major phosphorylated glycoproteins of milk is planned. The utility of the histochemical staining method for detecting phosphorylated proteins on gels has been demonstrated by the ability to detect previously uncharacterized phosphoproteins. It was also demonstrated previously that histochemical identification of the presence of phosphoproteins by their blue color within alveoli of lactating tissue correlated with the presence of blue staining phosphoproteins separated by electrophoresis on SDS-PAGE. The histochemical method was used also for detecting sialoglycoproteins by their blue-green color and approximately 1 nanomole of sialic acid in 5 μg of glycoprotein can be detected on gels. It is expected that it would be possible to correlate the presence of blue-green staining material in tissue sections indicative of such sialoglycoprotein production by mammary carcinoma cells within lymph nodes in metastatic disease with blue-green staining sialoglycoproteins on gels from proteins solubilized from those lymph nodes. No blue-green material is visible in sections from normal lymph nodes and it should be possible to distinguish

abnormal sialoglycoproteins made by cancer cells within lymph nodes on gels. This would be a different and perhaps simpler approach than that used currently. Such proteins could be isolated using their color tag and antigens could be made for RIA and used for detection of recurrent metastatic disease.

Stimulation by hydrocortisone of MMTV production and the necessity for the presence of hydrocortisone for the subsequent stimulation by prolactin of milk protein production has been demonstrated in this and other laboratories. The mechanism of this enhancement of protein synthesis has been correlated with enhanced mRNAs for virus and specific proteins. Unknowns are the critical receptor molecules or modifications of groups on those molecules, hormone interaction either with receptors on the cell membranes and subsequent transport to the nucleus and action at that level, and early events following hormone stimulation.

By comparing action of hydrocortisone and prolactin on protein production and modification in explants with or without hormone and/or virus it is hoped that a key action of hormone and virus on protein synthesis will emerge. It is expected that the virus function relevant to mammary tumor formation is expressed on many proteins being made by the mammary tissue. Since milk contains proteins modified by phosphorus and sialic acid and these modifiers have been shown to be key elements in recognition phenomena, we have begun to look at modification of these proteins as indicators of what may be happening elsewhere at the cell surface or the nucleus. The studies have as their aim to learn more about where and how steroids and prolactin act to stimulate breast development and milk production and how virus may interfere with this process, at what level and when and why abnormal enzyme and protein production occur that could be used as markers for pre-neoplastic disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 - CP - 04785-10 - LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Role of DNA Repair Mechanisms in the Etiology of Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.S. Day, III	Research Physical Scientist	LMC	NCI
OTHER:	C. Ziolkowski	Chemist	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study of defective repair of MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) produced damage was extended. One fifth (14/70) of the human tumor cell strains tested comprises a group of cell strains (Mer^-) that differs from the remaining 56 tumor strains (Mer^+) or from normal human fibroblasts (also Mer^+) in the following ways: 1) Inability to excise O^6-methylguanine from their DNA after being treated with MNNG; 2) relative inability to support the growth of MNNGdamaged adenovirus 5; and 3) sensitivity to being inactivated by MNNG or BCNU (Bis-chloroethyl-nitrosurea) in a reproductive way. The results may be relevant to uncovering the molecular reasons for success of certain bifunctional alkylating chemotherapeutic agents (BCNU, CCNU).

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To learn more about DNA repair mechanisms in human cells and about their role in carcinogenesis. In particular, to determine the nature of the defects both in human tumor cells and in cells from persons who are genetically predisposed to cancer. In addition, use human cell strains with characterized defects to study the mechanisms of action of carcinogens or suspect carcinogens in altering DNA.

Methods Employed: Using an adenovirus-host cell reactivation assay developed previously in this project, it has been possible to quantitate the deleterious effects of various chemical and physical treatments on the ability of the virus to initiate and sustain infection. The method involves establishing monolayer cell cultures which are then infected with treated or non-treated adenovirus. The infected cells are then incubated 12-20 days with feeding by means of periodic overlaying with a nutrient agar. Non-treated virions or those treated ones which have been "reactivated" by cellular repair mechanisms form countable plaques of dead, lysed cells. The plaquing efficiency of unirradiated adenovirus is approximately the same on all of the non-transformed human cell strains so far tested. It is 0.1 to 100-fold greater when human tumor lines of various types are used. To quantitate DNA base damage due to MNNG, methylated purines (released from DNA by combined acid, high temperature treatment of DNAs extracted from ^{14}C MNNG-treated cells) were separated by high pressure liquid chromatography.

Major Findings:

1) Fourteen of 70 cell strains prepared from human tumors were identified as less able to support the growth of MNNG-treated adenoviruses than the remaining 56. (For the sake of brevity of exposition, the phenotype so identified -- characteristic of the 14 strains -- is termed Mer^-. The phenotype shown by the other 56 is, therefore, called Mer^+.)

2) Seven of 11 strains of human fibroblasts transformed by SV40 also showed the Mer^- phenotype, whereas 33 strains of non-transformed human fibroblast strains (including the parent strains of four of the Mer^- SV40 transformed strains) were Mer^+.

3) Fibroblast cultures (of non-transformed appearance), prepared from two patients whose tumors gave rise to Mer^- cell strains, were found to be Mer^+.

4) A HeLa cell variant, found by others to be highly mutagenized by alkylating agents, was found to be Mer^-, whereas another variant, quite resistant to such mutagenesis, was Mer^+.

5) Eight strains having the Mer$^-$ phenotype were unable to remove O^6 methylguanine (produced in DNA by MNNG) from their DNA, whereas 5 Mer$^+$ strains were able to perform such repair.

6) In collaboration with Dr. Dominic Scudiero (now at FCRC), it has been determined that most cell strains having the Mer$^-$ phenotype are much more sensitive to being inactivated reproductively (colony forming ability) by MNNG than are Mer$^+$ cell strains. Similarly, Mer$^-$ strains are also sensitive to inactivation by BCNU.

Significance to Biomedical Research and the Program of the Institute:

The results of this project suggest the possibility that a fraction, one-fifth of all human tumors, is composed of repair-defective cells. Certain bi-functional alkylating agents (BCNU, CCNU) are known to be extremely effective against some human tumors and to be relatively ineffective against others, also indicating the possibility. It may be, therefore, that the molecular basis for the success of alkylation chemotherapy will soon be elucidated. A study of excision of O^6-methylguanine from MNNG-treated primary human tumor biopsies should provide evidence for the proof or disproof of the possibility. Physical, chemical, and viral carcinogens are all known to alter the structural integrity of the cellular genetic apparatus. An evaluation of the role of DNA repair and/or related mechanisms in conferring resistance or susceptibility to mutagenesis and carcinogenesis is an important facet in any overall program having as its goal the understanding of the molecular pathways which, when perturbed, give rise to carcinogenesis in humans. It is the long range goal of this project to determine whether or not the elucidation of genetic repair mechanisms is important to the understanding of carcinogenesis. It is expected that an understanding of human repair mechanisms in general will benefit many areas of biomedical research.

Proposed Course of Project: To pursue the goals outlined above in Objectives and to publish these results.

Publications

Day, Rufus S., III, and Ziolkowski, C.: An MNNG produced cellular response resulting in decreased survival of MNNG treated adenovirus 5. Proceedings of an International Meeting of the IGEGM: DNA Repair and Late Effects, Tel Aviv, May 1-5, 1978. In Press.

Day, Rufus S., III: Studying DNA repair using human adenoviruses 2 and 5: purification, plaque assay and inactivation. In Friedberg, E. C. and Hanawalt, P. C. (Eds.): Methods in DNA Repair, Marcel Dekker. In Press.

Day, Rufus S., III: The effects of 254mm ultraviolet light on E. coli bacteriophages lambda, T4, and OX174. Comprehensive Virology. In Press.

Day, Rufus S., III, and Chuck H.J. Ziolkowski: Four human brain tumor cell strains have deficient host-cell reactivation of N-methyl-N'-nitro-N-nitrosoguanidine damaged adenovirus 5. Nature 279: 797-799, 1979.

Day, Rufus S.,III, Ziolkowski, C.H.Z., Scudiero, D. A., Meyer, S. A., and Mattern, M. R.: Human tumor cell strains defective in the repair of alkylation damage. Carcinogenesis 1: 21-32, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05001-03-LMC

PERIOD COVERED
October, 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Role of DNA Damage and its Repair Mechanisms in in vitro Chemical Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Lo	Senior Staff Fellow	LMC	NCI
OTHER:	T. Kakunaga	Chief, Cell Genetics Section	LMC	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The DNA damages induced by carcinogens and their repair mechanisms are generall believed to be involved in cell transformation processes, but their direct role are poorly understood at the molecular level. Variants subcloned from BALB/3T3 A31 cone 1 have been found to exhibit different sensitivity to chemical-induced transformation. The purpose of this work is to determine whether the excision repair of benzo(a)pyrene (BP)-damaged DNA (i.e. BP-deoxyguanosine) of these cells is responsible for their different response to chemicals. With the three classes of variants studied, it has been found that all variants excised carcinogen-damaged DNA with extremely low efficiency. They are very much like the cells obtained from Xeroderma Pigmentosum (XP) patients with respect to repair deficiency.

Two immunological methods have been explored to assay the minute amount of BP-DNA formed in human KD and XP cells.

Project Description

Objectives: To study the biochemical mechanism of in vitro transformation by chemical carcinogens. With special attention to the role of DNA damages and repair mechanisms in this process. Current interests are: (1) To measure the excision repair of DNA damaged by BP in BALB 3T3 variants; (2) To explore more sensitive immunological methods for quantitation of BP-DNA adducts forme in human KD and XP cells.

Methods Employed: Cell culture, radioactive tracer, DNA isolation and measure ment techniques, column chromatography. Enzyme-linked immunosorbent assay (ELISA) is employed to study the competition of DNA samples with the antigen (i.e. BP-calf thymus DNA) for the limiting amount of antibody (made against BP-DNA, obtained from Dr. H. Slor) in the assay system. Nitrocellulose filter paper assay is employed to study the competition of DNA samples with the [^{3}H] BP-Col E1 DNA for antibody made against BP-DNA.

Major Findings: 1) The excision repair of BP-damaged DNA in 3T3 variants was measured by treating cells at the same concentration of BP used in the transformation studies (ie 0.5 M). These cells excised BP-DNA adduct with extremely low efficiency. More than 80% of the BP-deoxyguanosine adduct remained intact both in dividing and nondividing cells over 2 1/2 cell generations; 2) Very little difference in the excision repair capacity was observed between the highly transformable line (1-13) and the least transformable line (1-8), (12% excision vS 18% excision after 48 hrs postincubation of cells with BP free medium). Therefore, these cell lines are very much like XP cells with respect to deficiency in excisin repair; 3) ELISA and nitrocellulase filter paper assay are able to detect as low as 20 femtomoles of BP in the assays. Currently we are trying to modify the assay conditions for the purpose of reducing the nonspecific binding of antibody to the surface and improving the reproducibility of ELISA.

Significance to Biomdical Research and the Program of the Institute: (1) The results from this project will provide information about the mechanism of cel transformation, in particular the process of initiation of transformation by chemical carcinogens. The information obtained will be useful for the develo, ment of systems for assaying environmental carcinogens. (2) Once a more sens tive and quantitative immunological assay is established, one can determine the amount and types of carcinogen-DNA adduct formed in human cells under the same conditions used for transformation assays which is not possible at the present time.

Proposed Course of Project: To pursue the goals as outlined in objectives.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05012-03-LMC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structural and Functional Modification of DNA by Benzo(a)pyrene Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakefuda	Medical Officer	LMC NCI
OTHER:	Hiroshi Mizusawa	Visiting Fellow	LMC NCI
	Che-Hung Robert Lee	Staff Fellow	LMC NCI
	Patricia Madigan	Chemist	LMC NCI

COOPERATING UNITS (if any)

Richard J. Feldman DCRT, NIH

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The topic of the present study is the establishment of a highly sensitive and accurate method for detection of the structural modification of DNA caused by covalent and non-covalent interactions with benzo(a)-pyrene (BP) metabolites.

The effects of carcinogens bound to DNA on the functions of that DNA were studied by assaying replication, mutation, and repair of the DNA using both an in vitro replication system and a unique mutation assay system developed in this laboratory.

Several different clones of plasmid DNA which were mutated by a small number of BP metabolites are selected. Their transformability, restriction endonuclease map, and DNA base sequence are currently being studied in order to determine the location and chemical nature of DNA modification.

PHS-6040
(Rev. 10-76)

Project No. Z01-CP-05012-03-LMC

Project Description

Objectives: 1) To observe different forms of structural modification of DNA by carcinogen interactions; 2) to observe the genetic functions of DNA altered by covalent and non-covalent binding of carcinogens on DNA; and 3) to establish a system for analyzing the structure-function relationships of DNA modified carcinogens.

Methods Employed: 1) The BP metabolite, trans-7, 8-diol-9, 10-epoxide (DE-1) and closed circular DNA of plasmid pBR322, SV40, or pKO482, (the last a recombinant DNA consisting of genes of B-lactamase, galactose kinase, and origin for replication; gift from Dr. M. Rosenberg, NCI) were incubated together. The number of covalently bound DE-1 molecules was determined by radioactivity, UV absorbance, and fluorescence spectroscopy measurements. The structural modification of DNA was observed by electron microscopy, gel electrophoresis, and stepwise hydrolysis by specific nucleases. 2) For in vitro DNA synthesis, E.coli cells (strain YSI) were first lysed with lysozyme and 5% Brij 58, and then centrifuged at 25,000 rpm for 20 minutes. The resulting clear lysate was supplemented with ribo- and deoxy-nucleoside triphosphates, and used for in vitro replication of plasmid pBR322 DNA to which were bound various numbers of DE-1 molecules. The nascent DNA was labeled with ^{32}P-TMP and/or bromodeoxyuridine and analyzed by centrifugation in either an ethidium bromide-CsCl or a sucrose density gradient. 3) The SV40 DNA was incubated with DNA relaxation enzyme in the presence of different concentrations of BP metabolites. Intercalation of the metabolites in the DNA strand resulted in negative twisting of DNA after inactivating the enzyme and intercalating agent. The degreee of negative twisting, which depends on the number of intercalated molecules, was analyzed by agarose gel electrophoresis. 4) DE-1 was bound to pKO482 DNA (1-8 DE-1 molecules per DNA molecule). Transformation of the E.coli N99 strain (ampicillin sensitive and gal K- was assayed by formation of white colonies in ampicillin containing MacConkey galactose agar plates. DNA of mutant plasmids were cloned and were used for retransformation of N99 strain. The restriction endonuclease pattern of the cloned DNA was isolated from these strains, analyzed by gel electrophoresis, and DNA fragments were prepared and sequenced by the Maxim-Gilbert method.

Major Findings: Electron microscopic observation and gel electrophoresis revealed marked conformational changes, such as decrease in the number of superhelical turns, single and double strand nicks, and spontaneous self-excision of the DE-1-DNA base adduct. Portions of the DE-1 binding sites on DNA were denatured and became sensitive to endonuclease S1. In experiments using in vitro DNA synthesis as the assay, the elongation of the deoxypolynucleotide chain was blocked at the site of De-1 binding, whereas the initiation of DNA synthesis was nearly unaffected. Excision repair of the binding sites did not occur to a detectable degree in the cell extracts used in the experiments.

A variety of BP metabolites tested for potential intercalating activity using DNA relaxation enzyme assay showed no sign of intercalation except 7,8,9,10-tetrahydroxy BP, 7,8,9-trihydroxy BP, and 7,8-dihydroxy diol BP. Hydroxylation at the 7 and 8 positions seems to play an important role in the orientation of BP metabolites parallel to the plane of the DNA base pairs and in insertion of the aromatic pyrene moiety into the helix. In contrast, intercalation of the DE-1 molecule bound to the 2-amino group of guanine was highly restricted both in positioning and in movement of the DE-1 molecule. Thus the classic type of intercalation is not favored in this case. Conformational change observed was therefore considered to be a result of local denaturation and physico-chemical forces not now understood on the DNA helix.

Mutation of the pKO482 plasmid caused by covalent binding of DE-1 was found to be sensitive enough to detect a mutation caused by a single molecule of DE-1 binding to the gal K gene. The frequency of mutation by a single DE-1 binding per DNA is 4×10^4. Retransformation of N99 cells by mutated pKO482 DNA occurred in nearly all cases tested. The occurrence of spontaneous mutation incurred by pKO482 DNA in the transformation is below background level.

Significance to Biomedical Research and Program of the Institute: The binding of enzymatically activated metabolites of BP and subsequent modification of DNA is an important initial step of mutation and presumably of carcinogenesis. The highly sensitive and accurate assay system established by our study provides useful information for our understanding of the mechanism of mutagenic interaction with the genetic machinery of living systems. The *in vitro* DNA replication and mutation assay systems with plasmid DNA consisting of a few genes and a known number of carcinogen residues bound to it are useful tools for the investigation of the effect of carcinogen interaction on the genetic functions of DNA. A complete anaysis of the altered sequence of cloned DNA derived from mutated cells will elucidate the structure-function relationship of DNA modification.

Proposed Course of the Project: The effect of binding of various carcinogens on preassigned segments of DNA using recombinant plasmid DNA will be continued to be studied. The modified segment of DNA will be isolated and sequential change will be analyzed. Similar experiments will be carried out using SV40 DNA with eukaryotic cell systems.

Publications

Mizusawa, H. and Kakefuda T.: Inhibition of DNA synthesis *in vitro* by binding of benzo[a]pyrene metabolite diol-epoxide 1 to DNA. *Nature*, 279: 75-78, 1979.

Kimura, M., Otaki, N. and Kakefuda, T.: Characterization of cadmium-induced metallothionein in African green monkey kidney cells in vitro In Kagi, J.R. and Nordberg, M. (Eds): Metallothionein, Basel, Boston & Stuttgart, Birkhauser Verlag, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 - CP - 05068-02 - LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

HnRNA in Normal and Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. C. Peacock	Chief, Protein Section	LMC	NCI
OTHER:	T. Kakunaga	Chief, Cell Genetics Section	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.2	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Measurements of the amount of steady-state hnRNA and parameters characterizing its size distribution may be of value in understanding the biological properties of malignant cells. The populations of hnRNA found in several transformed and normal 3T3 cells and one normal and transformed human fibroblast line were compared. Uncompleted studies indicate that although the human and murine cells have characteristically different hnRNA populations, the normal and transformed cell lines are very similar.

Project Description

Objectives: To characterize a variety of neoplastic and normal cells as to their content of hnRNA, both with respect to amount and size parameters. Normal cells subject to perturbation as by hormones (e.g., mammary tissue, prolactin) will also be studied to learn whether hnRNA can play any role in cellular regulation.

Methods Employed: Cell culture, RNA, DNA analysis by electrophoresis, and RNA size distribution by thermal degradation (Project No. Z01-CP-04525-08-LMC).

Major Findings: 3T3 cells that were transformed by three different methods and their normal counterparts were found to be nearly alike with respect to their hnRNA. The similarity included size distribution and synthesis rate. Similarly, the hnRNA from normal and benzo[a]pyrene-transformed human KD cells were nearly indistinguishable.

These conclusions are tentative and are based on visual comparisons of hnRNA electrophoretic analysis. A more precise evaluation of the similarities and differences is in progress using the thermal degradation method to characterize the number and weight average distributions of size of hnRNA. Statistical analysis of this data will permit more definitive conclusions regarding similarities and differences.

Significance to Biomedical Research and the Program of the Institute: A common assumption is that the altered behavior of malignant cells is a result of mutation of the DNA. It is also believable, however, that malignant behavior could be a result of control aberrations alone. This work will help to discriminate among these hypotheses.

Proposed Course of Project: 1) Further detailed analysis of hnRNA of the cell types described above; 2) The effect of hormones and promoters will be investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 - CP - 05074-02 - LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Analysis of Factors That May Contribute to Estrogen Target Organ Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. R. Green Research Chemist LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to analyze factors which may contribute to the susceptibility of an estrogen target tissue, such as mammary gland, to cancer. Metabolic, dietary and environmental factors are studied. In vitro systems, such as organ culture and bacterial systems, are utilized to analyze possible mechanisms of action of specific compounds. Some lipsticks and their dyes were found to be mutagenic for the histidine-requiring tester strain, Salmonella typhimurium TA98.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Susceptibility of the mammary gland to cancer is governed by many diverse factors. Estrogens may promote carcinogenic processes initiated by environmental, dietary or genetic factors. Alternatively, estrogens may act by a feedback mechanism on the pituitary. Prolactin released by the pituitary may in turn promote carcinogenesis. Thus, both estrogens and prolactin have been viewed as promoters of the carcinogenic process initiated by other factors Certain chemical structural considerations, i.e., similarities to structures of known carcinogens, as well as animal studies and epidemiological considerations in women lead to an alternative hypothesis that under certain unusual metabolic conditions estrogen metabolites may become initiators. An in vitro system was chosen to assess whether estrogens and certain substances in the diet would show mutagenicity similar to that reported for known carcinogens such as benzo(a)pyrene and 7,12-dimethylbenzanthracene.

Methods Employed: Mutagenesis assay using histidine-requiring mutants of Salmonella typhimurium, organ culture, DNA binding studies, enzyme assays.

Major Findings: Estrogens, 7,12-dimethylbenzanthracene and some lipsticks and their dyes were assessed for mutagenicity using the histidine-requiring Salmonella tester strains developed by B. Ames.

Lipsticks contain dyes that have been provisionally approved for use under terms of the transitional provisions of the Color Amendment of 1960 pending completion of investigations for determination of their safety. Analytical data on results of long-term feeding tests in animals are as yet unavailable. Because such dyes are ingested and thus form part of the diet, it became of interest to determine whether the Ames test would reveal mutagenicitiy of lipsticks.

Twenty-four lipsticks of various shades and colors were tested for mutagenicity with the histidine-requiring tester strain Salmonella typhimurium TA98. Nine lipsticks were mutagenic without microsomal (S-9) activation. Dose-response effects were observed. Eight colorants listed as ingredients of the mutagenic lipsticks were tested with and without S-9. Drug and Cosmetic (D&C) Orange No. 17, a monoazo dye with two nitro groups, was highly mutagenic in the absence of S-9. The mutagenic effect was decreased or lost in the presence of S-9 prepared from livers of male non-inbred Sprague-Dawley rats given a single injection of Aroclor 1254. Eight lipsticks matched for ingredients other than dyes were tested. Two containing D&C Orange No. 17 were directly mutagenic. The mutagenic effect was decreased by the presence of S-9. Only D&C Orange No. 17 was sufficiently mutagenic without microsomal activation to account for the mutagenicity observed in these lipsticks. Lipsticks containing D&C Orange No. 17 and those labeled with the words "may contain" D&C Orange No. 17 should be suspected of being mutagenic for S. typhimurium TA98. This dye and 2,4-dinitroaniline, which may also be present, are potential health hazards.

Fifty μg of some lipsticks were as mutagenic without S-9 activation as 20 μg of 7,12-dimethylbenz[a]anthracene with S-9 activation. It is known that 2,4-

dinitroaniline, a breakdown product and possible contaminant of D&C Orange No. 17, is a toxic chemical and acts as an uncoupler of oxidative phosphorylation.

Estrogens activated by S-9 showed minimal mutagenicity, though statistically significant differences were observed in some experiments.

Significance to Biomedical Research and the Program of the Institute: Three types of compounds were assessed with the Salmonella/microsome test. One, the known animal carcinogen, 7,12-dimethylbenzanthracene, needed metabolic activation by S-9 to mutate the Salmonella tester strain. This chemical is very potent in causing breast cancer in female rats following feeding at low dose.

The dye D&C Orange No. 17 was highly mutagenic, but its carcinogenicity is not known.

Estrogens required metabolic activation by S-9 to demonstrate the very low mutagenic effects seen.

Breast cancer afflicts one in every eleven women in the United States. While dietary, environmental and genetic factors have been implicated, the primary cause of the disease seems far from clear. Since it is known from animal studies that certain types of ingested chemicals cause breast cancer, it would seem reasonable to try to assess very carefully chemicals that may be ingested whether the source be a "food" or a "cosmetic." Positive mutagenicity in the Ames test has shown high correlation with known animal carcinogens and for this reason is being used as a pre-screen for further testing in animals. Assessment of carcinogenicity of D&C Orange No. 17 and of 2,4-dinitroaniline awaits evaluation of results obtained by appropriate testing in animals. Chronic animal feeding studies of some current provisionally listed colors have been initiated elsewhere. No such studies are planned in this laboratory. 2,4-Dinitroaniline has been placed on a list of chemicals for possible testing for carcinogenicity in animals.

Proposed Course of Project: At present no formal causative relationship between mutagenicity and carcinogenicity has been demonstrated with respect to chemical carcinogens. The relationships established so far are correlative with respect to many known carcinogens. Investigations aimed at elucidating promoter and possible initiator effects of the steroids and prolactin on carcinogenesis of endocrine target tissue will continue. The mechanism of enhancement by prolactin of mammary carcinogenesis initiated by carcinogens, such as benzo(a)pyrene or 7,12-DMBA, is unexplained. By measuring adducts bound to DNA in the presence or absence of prolactin following exposure of explants to such carcinogens it might be possible to ascertain whether prolactin enhances binding to DNA and by that mechanism enhances tumor formation.

Publications

Green, M. R. and Pastewka, J. V.: Mutagenicity of some lipsticks and their dyes. J. Natl. Cancer Inst. 64: 665-669, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 - CP - 05075-02 - LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunological Investigation of Cytochrome P-450 Diversity in Human Monocytes and Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. M. Robie-Suh	Staff Fellow	LMC	NCI
OTHER:	H. V. Gelboin	Chief	LMC	NCI
	A. C. Peacock	Chief, Protein Section	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human lymphocytes and monocytes have been shown to exhibit mixed-function oxidase activity, a property which in many tissues and species is responsible for the activation of polycyclic hydrocarbon carcinogens. The levels of this activity vary among individuals and may be enhanced by culturing the cells with various inducers. In rat and rabbit tissues cytochrome P-450 is present in multiple forms and these forms differ in their substrate specificities. Multiple forms of cytochrome P-450 have not yet clearly been demonstrated in human tissues. Antibodies prepared against two forms of rat liver cytochrome P-450 have been found to inhibit mixed-function oxidase (i.e., benzo(a)pyrene hydroxylase) activity of human lymphocytes and monocytes. In this study the specificity of this inhibition is being investigated further with special attention being given to determining the relationship between antibody binding to cytochrome P-450 and inhibition of enzyme activity.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The aims of this study are: 1) to examine the nature of inhibition of cytochrome P-450-dependent mixed-function oxidase activity by antibody prepared against purified preparations of rat liver microsomal cytochrome P-450, and 2) to determine whether these antibody preparations might be useful in the examination of cytochrome P-450 diversity in human tissues.

Methods Employed: In this study antibodies against two types of rat liver microsomal cytochrome P-450, namely, the major form from 3-methylcholanthrene (3-MC)-treated rats and the major form from phenobarbital-treated rats, are being used as inhibitors of cytochrome P-450-dependent benzo(a)pyrene (BP) hydroxylation by uninduced and benz(a)anthracene (BA)-treated human lymphocytes and monocytes. Extent of inhibition of activity in lymphocytes and monocytes of a number of individuals is being determined and tabulated to see whether there are individual differences in sensitivity of enzyme activity to antibody inhibition and whether there are correlations between extent of inhibition and inducibility of the mixed-function oxidase (i.e., benzo(a)pyrene hydroxylase) and/or uninhibited activity of these cells. The BP metabolite profiles produced by the lymphocytes and monocytes in the presence and absence of antibody are being examined using high pressure liquid chromatographic separation techniques.

In addition, immunoprecipitation and immunoadsorption methods will be used in an attempt to determine the specificity of binding of the antibodies and to identify the moiety to which the antibody binds in these human cells.

Major Findings: Antibodies prepared against two forms of rat liver cytochrome P-450 inhibit cytochrome P-450-dependent BP hydroxylation by human lymphocytes and monocytes. The antibody fraction prepared against cytochrome P-450 from 3-MC-treated rats (IgG-MC) has greater inhibitory activity with both uninduced and BA-treated lymphocytes and monocytes than does the antibody prepared against cytochrome P-450 from phenobarbital-treated rats (IgG-PB). With IgG-MC the lymphocyte activity is more strongly inhibited than is the monocyte activity. The reason for this greater sensitivity of the lymphocytes remains to be determined. Although BA treatment elevates the BP hydroxylase activity of both lymphocytes and monocytes several-fold, this treatment has no significant effect on the degree of inhibition of this activity by either of the antibody preparations.

Preliminary evidence from studies of monocytes and lymphocytes of a dozen individuals indicates no relationship between extent of inhibition and fold induction or initial BP hydroxylase activity.

Immunoprecipitation experiments so far have not been useful in examining the binding specificity of the antibodies in the human cells due to 1) low levels of the enzyme in the cells and 2) a significant amount of non-specific binding that occurs upon incubation of the cells with the antibody preparations. However, modifications of the immunoprecipitation procedure to lower the non-specific binding could make use of this technique more fruitful.

Significance to Biomedical Research and the Program of the Institute: This study will provide information concerning the degree of similarity between cytochrome(s) P-450 of two species; namely, rat and human; further, it may facilitate the analysis and identification of multiple forms of cytochrome P-450 in human tissues, an accomplishment which would contribute greatly to our understanding of the role of enzyme multiplicity in the activation of polycyclic hydrocarbon carcinogens.

Proposed Course of Project: The specificity of binding of the IgG-MC in human monocytes and lymphocytes remains to be clarified and most of the work of this research period will be devoted to this problem.

Publications

Robie-Suh, K., Robinson, R., Gelboin, H. V., and Guengerich, F. P.: Antibody to purified rat liver cytochrome P-450 inhibits aryl hydrocarbon (benzo(a)-pyrene) hydroxylase activity of human monocytes and lymphocytes. Science, in press.

Guengerich, F. P., Wang, P., Mason, P. S., Robie-Suh, K., Robinson, R. C., and Gelboin, H. V.: Immunological cross-reactivity of rat liver and human cytochrome P-450. In Conney, A. H., Coon, M. J., Estabrook, R. W., Gelboin, H. V., Gillette, J. R., LaDu, B. N., and O'Brien, P. J. (Eds.): Microsomes, Drug Oxidations, and Chemical Carcinogens: Fourth International Symposium on Microsomes and Drug Oxidations. New York, Academic Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 - CP - 05081-02 - LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of DNA Synthesis in Diploid and Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. Mattern Senior Staff Fellow LMC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Nucleic Acid Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Differences in DNA replication in diploid and transformed human cells were studied. Transformed cells lacked the capacity to discriminate against the incorporation of 5-bromodeoxyuridine into DNA in the presence of thymidine, because 1) they are defective in the excision of misincorporated bases and/or 2) different DNA polymerases are used. Repair synthesis induced by UV or MNNG was inhibited more effectively by novobiocin or nalidixic acid than either replicative synthesis or repair synthesis induced by methylmethanesulfonate (MMS), suggesting that topoisomerase-mediated alteration of higher order DNA structure is required for DNA repair. However, cells could tolerate the disruption (by topoisomerase inhibitors) of such higher-order DNA structure for up to 24 hours and resume repair synthesis after UV. Replicative DNA synthesis in tumor cells defective in the repair of alkylation damage (Mer^-) was far more sensitive to MNNG than that in repair-proficient lines (Mer^+). Both Mer^+ and Mer^- cells could incise DNA after MNNG treatment, but the Mer- cells did so more slowly and did not restore their original DNA conformation, even after 24 hours.

Project Description

Objectives: To compare the mechanism and regulation of replicative and repair-type DNA synthesis in diploid mammalian cells and in transformed cells. Specific areas of study were: 1) Differences in the incorporation of DNA precursors by diploid and transformed human cells; differences in polymerases in vivo, 2) The relationship of higher-order DNA structure to DNA metabolism, 3) DNA metabolism in methylation repair-proficient (Mer^+) and deficient (Mer^-) human tumor cells.

Methods Employed: DNA synthesis was assayed in intact cells by measuring the incorporation of radioactively labelled precursors (thymidine or bromodeoxyuridine) into TCA-insoluble material. BUdR discrimination was determined according to published procedures (Mattern, M., Exp. Cell Res., 1980, in press). Repair DNA synthesis was ascertained according to the BND-cellulose method of Scudiero et al (Mutation Res. 29, 373-388, 1975). Survival after UV, novobiocin, or nalidixic acid treatment was determined according to published procedures (Day et al, 1980). In situ treatment of cells with DNA polymerase inhibitors was carried out by incubating cultures (labelled previously with ^{14}C-thymidine for approximately 1 cell generation) for 1 hour in the presence of growth medium containing appropriate amounts of inhibitor; in the case of heat treatment, cultures were placed in a 45° water bath for various periods of time as described (Mattern, 1980, Exp. Cell Res., in press). Percentage of control DNA synthesis (nascent ^3H-thymidine incorporation divided by ^{14}C-parental DNA) was determined for each treated culture. The sedimentation of nucleoids in neutral sucrose gradients (15-30%) was as decribed (Mattern and Painter, Biochim. Biophys. Acta 563, 293-305, 1979). The fraction sedimented was expressed as the ratio of the peak fraction number to the total number of fractions in the gradient.

Major Findings: 1. Differences in DNA replication in diploid and transformed cells. a) Discrimination against BUdR by diploid cells; lack of discrimination by transformed cells.

The suggestion that transformed cells fail to discriminate against BUdR because they use a different complement of DNA polymerases was tested by treating cells with cytosine arabinoside [araC] (a specific inhibitor of α-type DNA polymerases) and with heat (45°C) or dideoxythymidine (specific for β-type DNA polymerases). Replicative DNA synthesis in the transformed cell lines tested was more sensitive to 45° treatment and dideoythymidine than the diploids and less sensitive to araC as well as N-ethylmaleimide (Mattern, 1980, Exp. Cell Res., in press). These results are consistent with an alteration in the DNA replication in transformed cells such that β-type polymerases are used more extensively than in diploid cells; this interpretation is supported by the further finding that discrimination against BUdR in diploid cells seems to depend on the α-polymerase. Initial attempts to measure excision of incorporated ^{14}C-BU indicated that diploid cells may be able to excise the misincorporated analogue, whereas transformed cells may not. These studies will be expanded to include more cell lines and more complete kinetics. Biological

methods will be employed (i.e., survival of BU-labelled adenoviruses) as an alternate and perhaps more sensitive means of deecting BU excision (work with R.Day). 2. Relationship of higher-order DNA structure to replicative and repair-type DNA synthesis.** (work with R. Day and D. Scudiero). a) Studies with bacterial DNA topoisomerase inhibitors (novobiocin and nalidixic acid) were extended**. Novobiocin and Nalidixic acid both inhibit primarily DNA replication (RNA and protein synthesis are also blocked, but less efficiently). Both replicative and repair-type DNA synthesis were inhibited; the most sensitive process (to both novobiocin and nalidixic acid) was repair DNA synthesis induced by either UV or MNNG (work with D. Scudiero). Repair synthesis induced by methylmethane-sulfhonate (MMS) was as sensitive to novobiocin and slightly less sensitive to nalidixic acid than replicative DNA synthesis. The latter agent, unlike UV light or MNNG, produces extensive strand breakage of DNA. These results suggest that one step of DNA repair may be the participation of DNA topoirsoimerases in altering the higher order structure of DNA (supercoiling, etc.) to make the damaged DNA sites more accessible to repair proteins. DNA strand breaks could perform this function in part, thereby obviating the requirement for topoismerase.

The finding that novobiocin treatment for up to 24 hrs did not enhance significantly the killing effect of UV light prompted an investigation of repair synthesis in cells that were UV irradiated and incubated with novobiocin for 24 hours and then in fresh drug-free medium for various times before repair incubation with ^{3}H-thymidine. After the cells were released from the novobiocin block, DNA repair synthesis took place within approximately 3 hrs; the total amount of incorporation per cell was equivalent to that occurrin under conditions of UV repair that was not inhibited by novobiocin. The nature of this quiescent state induced by topoisomerase inhibitors and the effect of the inhibitors on the initial (pre-polymerisation) steps of DNA repair remain to be investigated.

3. DNA metabolism in methylation repair-proficient (Mer^{+}) and deficient (Mer^{-}) tumor cells (work with R. Day). Mer^{+} and Mer^{-} tumor cell lines have been described (Day, R. & Ziolkowski, C., Nature 279, 797-799, 1979). These cultures and diploid human fibroblasts (which are also Mer^{+}) were studied to determine: (a) The effect of the alkylating agent MNNG on replicative DNA synthesis immediately after and 24 hours after its incubation with the cells for 60 min. In the Mer^{+} cultures, there was less inhibition initially at a given concentration of MNNG than in Mer^{-} cells; 24 hours later, there was some recovery of DNA synthesis. Besides being much more inhibited in DNA replication initially, Mer^{-} cells were even more sensitive to MNNG 24 hours later. This result is in contrast with that of D. Scudiero (Day, R. et al, Carcinogenesis 1, 21-32, 1980), i.e., that MNNG-induced repair-type DNA synthesis is greater in amount in the Mer^{-} cells. The question of the relationship of repair synthesis to survival of damaging treatment will be pursued. (b) The kinetics of restoration of sedimentation properties of

Project Description

nucleoids in cells treated for 60 min. with MNNG. The difference between Mer^+ and Mer^- cell lines in the amount of MNNG - induced repair synthesis (See above) suggests that the relation between the total cpm/ug DNA incorporated into repair patches and survival following treatment of cells with an agent th damages DNA is not a simple one. The incision step of repair was investigated using neutral sucrose gradient sedimentation (see above). Preliminary results indicate that after 100 uM MNNG treatment both Mer^+ and Mer^- tumor cells are able to incise the damaged DNA. Diploid human and Mer^+ tumor cells perform this step more rapidly than the Mer^- cells and after approximately 2 hrs their nucleoids sedimented nearly as rapidly in the neutral sucrose gradients as tho from untreated control cultures, consistent with the initial relaxation and subsequent restoration of a higher-order DNA structure (which makes the DNA at first less and then more compact). A restoration of this magnitude was not observed with Mer^- cells within 24 - 48 hours. These studies will be extended to include a greater number of cell lines, measurements of reapir "patch sizes after treatment with either MNNG or other agents (to the latter of which Mer^+ and Mer^- cells are equally sensitive) (work with R. Day/D. Scudiero), and determination of the effects of specific DNA polymerase inhibitors upon replicative and repair-type DNA synthesis in Mer^+ vs Mer^- cells.

Significance to Biomedical Research and the Program of This Institute: The way in which cellular DNA is replicated and maintained between duplications is altered in transformed cells. Understanding these alterations at the molecular and biological levels through comparative studies of DNA metabolism (replication and repair) will contribute to a greater understanding of the mechanism of malignant transformaton as well as the "usual"mechanisms and regulation of these functions. The latter is of general biomedical interest.

Proposed Course of Project: To continue the studies in the three general areas described and to communicate the results through appropriate professiona vehicles.

Publications

Day, R., et al.: Carcinogenesis I, 1980 , pp. 21-32.
Mattern, M.: Exp. Cell Res., in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 - CP - 05083-02 - LMC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

An Immunological Approach to Study DNA Damage and Repair by Irradiation and Chemical Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. Slor Visiting Scientist LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies against DNA modified by benzo(a)pyrene diol-epoxide I (BP-DNA) were raised, purified and studied in several ways. These antibodies did not react with double-or single-stranded DNA or with BP-RNA or BP-protein. Such antibodies were used in initial attempts to detect repair of BP-DNA in treated cells by fluorescence assay, to detect BP residues on DNA by electron microscopy, to detect BP residues on the DNA of gently permeabilized cells. The feasibility of a monoclonal system for producing such antibodies is under investigation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives are: 1) To learn about DNA repair mechanisms in human cells with an emphasis on repair of carcinogen damage; 2) To determine the role of DNA repair in carcinogenesis with an emphasis on developing radioimmunoassays to detect specifically various types of adducts in different cell types or organs; and 3) To correlate the finding of damage and repair of given carcinogen adducts to the sensitivity of these cells or organs to malignant transformation by the same adducts.

Methods Employed: Preparation of antibodies: DNA is modified either by irradiation or treatment with chemical carcinogens by established methods. The DNA is purified and complexed with methylated bovine serum albumin (MBSA) at equimolar concentrations, mixed with complete Freund's adjuvant, and injected intramuscularly into a rabbit, usually at a concentration of 1 mg/ml. The immunization procedure may vary but involves at least one injection per week for at least 8 weeks. Sera from bled rabbits are tested periodically for reactivity. When reactive sera are obtained, a lag period of 2-3 weeks without immunization is allowed. This is followed by two weekly immunizations (boosters). Reactive antisera are then purified to obtain the immunoglobulins from which the specific antibodies may be further purified by affinity chromatography employing a cellulose-BP DNA column to which the specific antibodies bind and may eventually be eluted.

The basic radioimmunoassay employed in modified native DNA studies utilizes the fact that native DNA is not retained on nitrocellulose filters, whereas if antibody is bound to the DNA, the DNA-antibody complex is retained on the filter. Using labeled DNA (^{3}H or ^{14}C) in such assays, the radioactivity retained on the filters serves as a measure of antibody reactivity. Other methods involve the use of anti-rabbit antiody as a third layer (second antibody method), and if the second antibody is radioactively labeled, the radioactivity bound to the DNA can serve as a direct measure of the number of antibody molecules bound to the modified sites of the DNA molecule.

Major Findings: We have successfully raised antibodies against AAAF-DNA adducts and against benzopyrene-DNA adducts. We are attempting to raise antibodies vs psoralen-DNA adducts, novobiocin, and MNNG adducts.

The antibodies against BP adducts were purified by ammonium sulfae precipitation, G-25 sephadex gel filtration, DEAE-cellulose chromatography, and by affinity chromatography on a sepharose-anti:rabbit IgG column. Serial dilutions of these antibodies established that they retain 50% reactivity at 1:20,000 dilution. They are absolutely specific for DNA-BP, do not react with RNA-BP or protein BP, or with unmodified DNA, either native or heat-denatured. Competition experiments demonstrated that denatured DNA-BP inhibits the binding of plasmid ColE1 ^{3}H BP-DNA to the antibody 10 to 15 times more than native DNA-BP. This may be explained either by assuming that the antibodies have greatly increased affinity towards BP-adducts in denatured DNA compared to native DNA, or alternatively, by assuming that there are more BP-adducts that are accessible to the antibodies in denatured DNA compared with native DNA.

Project Description

Normal and XP cells treated with BP-diolepoxide were fixed at various times after treatment, treated with Abs and subsequently with Fluoroscein-tagged anti-rabbit IgG and viewed by fluorescence microscopy. The only fluorescent nuclei were those of BP-treated cells. Nuclei from untreated cells showed no fluorescence. Brighter fluorescent spots over the nuclei were identified from phase-contrast pictures as nucleoli or heterochromatin. After 12 h and 24 h nuclei were less fluorescent. (Work done in DCCP in collaboration with M. Bustin, LMC, CIP, DCCP.)

ColE1-BP or ColE1 (control) were treated with BP antibodies and anti-rabbit IgG taged with Ferritin, and processed for electronmicrocopy. Only BP-DNA had well defined black round, spots (Ferritin-tagged Abs). No such spots were seen in control DNA.

Permeable Cells:

CHO cells were treated with BP-diol epoxide-I permeabilized by detergent, treated with BP-antibodies and fixed. Treatment of the fixed cells with fluorescent anti-IgG or protein A demonstrated the presence of IgG inside the cells. (Collaboration with M. Mattern, LMC, CIP, DCCP.)

If we should be able to introduce specific antibodies by microinjection or by fusion under conditions that allow cell survival, we may be able to block the excision of specific types of adducts by the use of specific antibodies, and follow the biological consequences to the cell. Experiments using red blood cells ghosts,filled with antibodies, sealed, and fused with carcinogen (BP)-treated cells are in progress.

Enzyme Immunoassay:

We have adapted enzyme immunoassay for determining DNA-BP antibodies.

Microtiter plates were coated with BP-DNA, treated with BP-antibodies, and finally with Alkaline Phosphase-bound anti rabbit IgG. The final step was incubation with an alkaline phosphatase substrate. The yellow color produced is correlated with the initial titer of antigen: Ab complex.

Using this assay will enable us to identify hybridoma cultures that produce anti BP-DNA antibodies in an attempt to produce monoclonal antibodies to specific types of BP adducts. This project has begun. The initial results are encouraging.

Significance to Biomedical Research and the Program of the Institute:
The availability of a radioimmunoassay to study both the formation and removal of damaged sites in treated DNA will enable us to correlate the cellular capacity to remove DNA damage, or cellular sensitivity to being damaged by carcinogenic agents, to the established sensitivity

of these cells to transformation. In addition, the radioimmunoassay may enable us to study specifically different types of adducts formed and removed from the DNA.

Certain human inherited diseases are associated with a high incidence of cancer (e.g., Xeroderma Pigmentosum, Ataxia Telangiectasia, Bloom's syndrome, Fanconi's Anemia). Cells from some such patients are sensitive to certain agents (i.e., chemical carcinogens or irradiation) compared to normal cells, and have reduced levels of DNA repair. Specific antibodies to various types of damaged DNA may help to identify precisely the initial type of damage each type of genetically affected cell fails to repair and to correlate this finding to the high incidence of cancer in donors affected by these inherited diseases.

Proposed Course of Project: To pursue the goals outlined above in Objectives, publish the results, and make the antibodies available to the scientific community.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05086-02-LMC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of Benzo(a)pyrene Metabolism in Human Cells: Studies with Monoclonal Antibodies to Cytochrome P-450's.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sang Shin Park	Visiting Associate	LMC	NCI
OTHERS:	Harry V. Gelboin	Chief	LMC	NCI
	Sun-Ju (Kwon) Cha	Guest Research Pharmacologist	LMC	NCI
	Haruko Miller	Chemist	LMC	NCI
	Richard Robinson	Biologist	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Benzo[a]pyrene (BP) is oxidized by mixed function oxidases (aryl hydrocarbon hydroxylase, AHH) as an initial step for the formation of an ultimate carcinogen, 7,8-diol-9,10-epoxide with the function of epoxide hydratase (EH). Human monocytes, lymphocytes and human lung cell line, A549 were grown in the presence of benzo[a]anthracene (BA) as an inducer. Microsomes were prepared from human liver and placenta of heavily smoking women. When the cells were tested for AHH and EH activity, the levels of enzymes were 2-4 times of the uninduced cell activities. The human placenta microsomes also retained high AHH activity. However, the AHH activity of human placenta microsomes was reduced by 21%, monocytes induced by BA by 5.4% and rabbit microsomes induced by phenobarbital (PB) by 27% at 1:2 dilutions by preincubating with monoclonal antibodies to liver microsomal cytochrome P-450 LM2 of rabbits induced by PB. The purpose of this work is to investigate the appearance of a specific predominant form of isozymes involved upon induction using monoclonal antibodies as tools.

Project No. Z01-CP-05086-02-LMC

Project Description

Objectives: The pretreatment of rodents with small amount of polyaromatic hydrocarbon (PHA) or intraperitoneal injection of AHH inducer decreased or inhibited the tumor formations in lung and mammary glands. On the other hand, PHA's are metabolically activated to cytotoxic, mutagenic, and carcinogenic metabolites in procaryotic and eukaryotic cells. Therefore, either one of the effects could occur upon the induction of mixed function oxidase (AHH) with PHA: detoxification or the formation of 7,8-diol-9,10-epoxides leading to carcinogenesis.
The balance could be determined by the presence of a predominant form of P-450 induced by a specific inducer or by the stero-specificity and substrate selectivity of the enzyme because rabbit microsomal cytochrome P-450 LM2 was the predominant from induced by phenobarbital and P-450 LM4 was another predominant form induced by beta-naphtoflavone. The present objective is to investigate the predominant form of enzymes in human cells and microsomes induced or derived from different sources with the observation of cross-reactivity of monoclonal antibodies to rabbit liver microsomal cytochrome P-450 LM2 and LM4 and cytochrome P-450's of human sources.

Methods Employed: Monocytes and lymphocytes were isolated from human blood. Human lung cell line, A549 was the established cell line from a lung tumor. Microsomes were prepared from human liver and placenta of heavily smoking women. Each cell line grown in the presence or absence of benzo[a]anthracene as an inducer and microsomes were testd for AHH and EH activity after preincubation with monoclonal antibodies. AHH was determined by measuring the amount of BP conversion to phenolic products equivalent to 3-OH BP. EH activity was measured by differential extractjion of BP-dihydrodiol, the product of enzyme reaction on BP-4,5-epoxide. Monoclonal antibodies were prepared by fusing myeloma cells and spleen cells derived from Balb/c female mice immunized with a specific antigen.

Major Findings: 1) Human blood monocytes, lymphocytes and A549 lung cells were inducible for AHH and EH activity in the presence of benzo [a]anthracene. 2) The levels of AHH activity of placenta microsomes were varied with the individual placenta but relatively very high. 3) The AHH activity of placenta microsomes of heavily smoking women was reduced by 21% while the AHH activity of monocytes induced by benzo[a] anthracene was reduced by 5.4% by preincubation with monoclonal antibodies to purified cytochrome P-450 LM2 of rabbits induced with phenobarbital. 4) The AHH acitivity of rabbit microsomes was inhibited by 27% with the monoclonal antibodies.

Significance to Biomedical Research and the Program of the Institute: Benzo[a]pyrene is oxidized as an initial step by the microsomal mixed function oxidases which contain multiple forms of P-450. Human individuals show difference in susceptibility to carcinogens reasons for

which are unknown. The presence of multiple forms of enzymes, double edged sword metabolism of BP and differences in individual susceptibility to carcinogens, all might have correlations leading to carcinogenesis. Monoclonal antibodies are specific to a specific antigenic determinant. Therefore, the analysis of the presence of the predominant form of P-450 upon induction by different inducers would be helpful in understanding the BP metabolism with respect to regulation as well as chemical carcinogenesis.

Proposed Course: Benzo[a]pyrene is oxidized by the mixed function oxidase (AHH) and metabolically related enzymes to more than forty oxigenated metabolites which include simple epoxides, phenols, quinones, dihydrodiols, diol epoxides and water soluble conjugates of glutathione, glucruonide and sulfate. Therefore, all enzymes involved in the BP metabolism have to be analyzed biochemically and immunologically using monoclonal antibodies for the presence of predominant forms of enzymes and any feed back control in BP metabolism.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05089-02-LMC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Role of Rat and Human Liver Epoxide Hydratase on Cytochome P-450 LM_4 and Cell Metabolism of ^3H (-)t-7,8-Dihydroxy-7,8-Dihydrobenzo(a)pyrene and Binding to DNA and Autocatalyzed Protein Binding.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.M. Gozukara	Visiting Scientist	LMC NCI
OTHERS:	H.V. Gelboin	Chief	LMC NCI
	R.C. Robinson	Biologist	LMC NCI
	F.P. Guengerich	Associate Professor of Biochemistry, Vanderbilt University, Tennessee	
	M. J. Coon	Professor of Biochemistry University	

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	7.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of purified rat liver epoxide hydratase on the disposition of reactive metabolites formed from ^3H(-)t-7t-7,8-diol PB by either purified mixed-function oxidase system or human monocytes, lymphocytes and Fisher rat liver (TRL-2) cells were studied. The binding of reactive metabolites such as diol epoxide I and II to DNA and to purified P-450 LM_4, NADPH-cytochome c (P-450) reductase, epoxide hydratase and BSA were examined. The hydrolysis product of diol epoxide I and II were analyzed by high pressure liquid chromatography (HPLC).

In the second part of the project, the role of Human liver epoxide hydratase were examined. ^3H(-)t-7,8-diol PB was used as a substrate to generate diol epoxide I and II in the incubation medium, by using Control and BA-preinduced human monocytes, lymphocytes, Fisher rat liver (TRL-2) cells, and Buffalo rat liver (BR cells. The binding of reactive metabolites to added and intact cellular DNA was determined. The epoxide hydratase was added either to incubation mixture or to tissue culture media and inhibition to DNA binding was determined.

Project No. Z01-CP-05089-02-LMC

Project Description

Objectives: To determine the metabolism of $^{3}H(-)$t-7,8-diol BP and covalent binding of reactive metabolites to added DNA and intact cellular DNA in tissue culture media. The purpose of this project is to understand detoxification or activation role of human liver and rat liver epoxide hydratase.

Methods Employed: Fluorospectrophotometry, high pressure liquid chromotography, slab gel electrophoresis, radiography, tissue culture techniques and Ames test.

Major Findings: The reactive metabolites formed form $^{3}H(-)$ t-7,8- diol BP was found to bind covalently to DNA. The binding of metabolites to DNA is a linear function of cytochrome P-450 LM_4 content. Addition of epoxide hydratase reduced the binding of metabolites to DNA. Human monocytes, lymphocytes and Fisher rat liver (TRL-2) cells also catalyze binding of $^{3}H(-)$t-7,8-diol BP to added DNA. The cells that are pre-induced by benzanthracene (BA) exhibit greater levels of DNA binding than control cells. In all cases addition of epoxide hydratase to the medium reduced the amount of reactive metabolites bind to DNA. The analysis of diol epoxide I and II hydrolysis by high pressure liquid chromatography showed that DNA had an effect on the stereoselectivity of diol epoxide hydrolysis. The addition of epoxide hydratase reduced the formation of tetrols I-1, I-2 and II-2 and increased tetroll II-1 formation. This result indicates that the trans addition of - OH group to position. C-10 may be catalyzed by epoxide hydratase. Addition of DNA to incubation medium reduced protein binding in the presence or absence or absence of epoxide hydratase. This indicates that DNA is competing with proteins for reactive metabolites. We have also reported that diol epoxides formed from $^{3}H(-)$ t-7,8-diol BP react directly with the enzymes responsible for their production in the reconstituted system. The addition of epoxide hydratase to the incubation reduces the covalent binding to all purified proteins.

Human monocytes, lymphocytes, Fisher rat liver (TRL-2) cells, and Buffalo rat liver (BRL) cells also catalyze binding of $^{3}H(-)$ t-7,8-diol BP to added DNA. Addition of human liver epoxide hydratase reduced DNA binding in all cases. We reported that addition of $^{3}H(-)$t-7,8-diol BP to tissue culture media resulted the binding of metabolites to intact cellular DNA. When $^{3}H(-)$ t-7,8-diol BP and epoxide hydratase were added of the same time cellular DNA binding was also inhibited in Human monocytes and lymphocytes. Inhibition of DNA binding by added hydratase indicates that the hydratase interacts stereospecifically with the diol epoxides, binding them covalently altering their mode of hydrolysis and apparently reducing their activity as carcinogenic intermediates that bind to DNA.

Significance to Biomedical Research and the Program of the Institute: Metabolism of $^{3}H(-)$t, 7,8-diol by purified reconstituted and cellular

mixed-function oxidase system and binding of reactive metabolites to added and also cellular DNA and proteins is an essential step toward the understanding of chemical carcinogenic action. Binding of reactive metabolites to DNA can lead to cell death, mutation or transformation to a cancer cell, if such DNA damage is not properly repaired before cell division. DNA and protein binding reduced by addition of epoxide hydrotase. The P-450 protein is believed to be the terminal oxidase for diol epoxide formation and the diol epoxide bound may be more related to the amount formed rather than to its disposition by the hydratase. The addition of epoxide hydratase to the tissue culture media reduced DNA binding in human monocytes and lymphocytes. These findings indicate that epoxide hydratase may play an important role in detoxification in living cells.

Proposed Course of Project: To determine the production of diol epoxide I and II in incubation medium by using purified reconstituted mixed function oxidase system and different kind of cells and binding of reactive metabolites to added DNA and intact cellular DNA. To understant the role of human and rat liver epoxide hydratase in the activation and detoxification of (-) t-7,8-diol BP and try to understand toxicity, mutagenicity and carcinogenicity of the metabolites.

Publications

Gozukara, E.M., Belvedere, G., Deutsch, J., Coon, M.J., Guergerich, F.P., and Gelboin, H.V. Autocatalyzed covalent binding of $^{3}H(-)$t-7,8 diol benzo [a] pyrene to mixed-function oxidase enzymes and DNA: The role of epoxide hydratase. Coon, M.J., Coney, A.H., Estabrook, R.W., Gelboin, H.V., Gillette, J.R. and O'Brien, P.J. (Eds): Fourth International Symposium on Microsomes and Drug Oxidations. New York, Academic Press (In Press).

Gozukara, E.M., Belvedere, G., Deutsch, J., Coon, M.J., Guergerich, F.P., and Gelboin, H.V. The role of epoxide hydratase on cytochrome P-450 LM_4 and cell metabolism of $^{3}H(-)$ t-7,8-dihydroxy-7,8-dihydro benzo[a] pyrene and binding to DNA autocatalyzed protein binding. Molecular Pharmacology (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01- CP -05109-01 - LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Interaction of Chemical Carcinogens with the Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Bustin	Visiting Scientist	LMC	NCI
OTHER:	M. M. Seidman	Senior Staff Fellow	LMC	NCI
	P. D. Kurth	Staff Fellow	LMC	NCI
	L. Einck	Guest Worker	LMC	NCI
	H. Slor	Visiting Scientist	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interaction of chemical carcinogens with the genome of a target cell is being studied by: 1) examining the binding of benzo[a]pyrene to SV40 minichromosomes, 2) examining the binding of benzo[a]pyrene to polytene chromosomes, and 3) developing monoclonal antibodies to DNA which has been modified by diol epoxide I. An antibody detection technique which allows detection of one carcinogen adduct per molecule of DNA has been developed. Homogenates of Chironomus thummi metabolize benzo[a]pyrene. Diol epoxide I binds to polytene chromosomes in a random fashion. The carcinogen does not bind to a selected genetic locus. Mice immunized with diol epoxide I-modified DNA produce antibodies which will react with the immunogen, but not with unmodified DNA.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To study the mechanism of the interaction of chemical carcinogens with the genome. To understand the influence of the structure and packing of the chromatin fiber on the binding of a carcinogen to its target.

Background and Research Strategy: The interaction of a carcinogen with the genome of a target cell seems to be the critical event leading to transformation. While it is known that certain chemical carcinogens interact with various constituents of the genome, it is not clear how the structure of chromatin and chromosomes influences the binding of the carcinogen to its target.

A program designed to elucidate some facets of these questions has been recently initiated. The program involves three different, albeit interrelated, experimental approaches.

The first approach involves the use of the SV40 minichromosome as a well-defined model for the eukaryotic chromosome. The major question under consideration is whether all reactive nucleotides are uniformly accessible to the carcinogen. This major question can be subdivided into the following more specific questions: 1) Is there a "hot spot" for carcinogen binding? 2) Is there variability between types of sequences, i.e., coding vs. regulatory, transcribed vs. non-transcribed? 3) Is there variability between coding and non-coding strand accessibility? 4) Is there difference between nucleosomal or spacer DNA?

The SV40 chromosome is an ideal system to study these questions since it is a well-defined system whose DNA sequence has been established.

The second approach involves the use of polytene chromosomes. These giant chromosomes are a convenient system to study various structure-function relations in the genome. Specific genes can be visualized by conventional light microscopy and the chromosomes can be experimentally manipulated so as to induce and visualize transcriptional activity in defined genetic loci.

While the SV40 system allows investigation at the DNA sequence level, the polytene chromosome system allows investigation of similar questions, but on a more macroscopic level. The following questions will be investigated: 1) Are there "hot spots" for carcinogen binding? 2) Does the carcinogen bind to a few selected genes? 3) Does a change in the macromolecular structure of a genetic locus affect the binding? 4) Is there a difference in the accessibility of the carcinogen between transcribed, condensed and uncondensed regions of the chromosome?

The third approach involves the development of immunochemical techniques to study various aspects of damage to the genome induced by the carcinogens and its subsequent repair. It is planned to obtain monoclonal antibodies specific to DNA which has been modified by diol epoxide I.

Methods Employed: An encapsidation mutant growing in permissive cells is used as the target for the carcinogen. The carcinogen is either benzo[a]pyrene or its metabolite, benzo[a]pyrene 7,8-diol-9,10-epoxide I. Detection of the binding of the carcinogen to DNA is accomplished by two methods. The first is to use benzopyrene as a depurinating reagent and use accepted DNA sequence techniques to pinpoint the location of the damaged base. The second involves transfer of the modified DNA fragment to DBM paper and visualize the location of the modified DNA fragment by accepted immunochemical techniques.

For the polytene chromosome system, salivary glands are obtained from the 4th instar of Chironomus thummi. Polytene chromosome squashes are prepared by conventional techniques. The squashes are reacted with ^{3}H-diol epoxide I and the location of bound carcinogen visualized by autoradiography. The grain density in the various regions of the polytene chromosomes is determined semi-automatically using an Artek Model 880 micro/macro counting system.

For determination of the in vivo binding sites, larvae are grown in petri dishes in 10% DMSO and about 1 mCi ^{3}H-benzopyrene. After 1-24 hour incubation the larvae are washed and squashes of salivary glands are processed for auto-radiographs.

The presence of a mixed-function oxidase system in Chironomus is detected by difference spectroscopy and by fluorometric assay for aryl hydrocarbon hydroxy-lase activity.

The ability of Chironomus to metabolize benzo[a]pyrene is determined by high performance liquid chromatography using a Whatman Partisil ODS-2 column with a linear 60-100% methanol-water gradient.

For monoclonal antibodies, female BALB/c mice, 6-8 weeks old, are immunized with 0.2 ml containing 100 μg of DNA modified 1.5% with diol epoxide I. An additional 5 mice are injected in the same manner except that the DNA is complexed with methylated BSA. After appropriate boosting, the presence of specific antibodies is detected by a filter assay technique.

Antibody-producing mice are sacrificed and their spleens fused with myeloma cells (1×10^{9} spleen cells with 1×10^{8} myeloma cells) using 50% polyethylene glycol (1000 MW) for 1 minute. The myeloma cell line (P3 x 63 Ag 8g, BALB/c origin, MOPC, kappa producer) is maintained in DMEM high glucose, high bicarbo-nate, 10% fetal bovine serum, 10% horse serum + additional glutamine + azo-guanine. Hybrid cells are selected by a two-week incubation in media contain-ing HAT. Fibroblasts are eliminated by repeated transfer. Hybrid cells are screened for secretion of both specific and non-specific antibody by a solid-phase enzyme-linked immunoassay.

Major Findings: This project has been recently initiated. So far it was possible to establish most of the experimental systems. The following has been accomplished: 1) A cell culture system required for the growth of SV40 has been established. The chromosome formed during the infection of permis-sive cells with an encapsidation mutant of SV40 has been isolated and

characterized. 2) Conditions have been found for reaction of 7,8-diol 9,10-epoxide with the viral DNA *in vivo* so that one carcinogen per viral chromosome is covalently bound. 3) An antibody detection technique has been developed. This method allows the display of a restriction pattern of carcinogen-modified DNA by gel electrophoresis. The separated fragments are transferred and covalently bound to DBM paper. The carcinogen-modified fragments are detected with antibody ^{125}I-protein A and autoradiography. It is possible to detect as little as one carcinogen molecule per DNA fragment. 4) Homogenates of *Chironomus thummi* larvae contain a constitutively high level of aryl hydrocarbon hydroxylase activity. Benzo[a]pyrene is metabolized by larvae homogenates to yield a metabolic profile similar to that observed by inducible mammalian cells 5) Radioactive benzo[a]pyrene 7,8-diol 9,10-epoxide I binds to polytene chromosome squashes. The binding of the carcinogen to both formaldehyde-fixed and unfixed chromosomes is time- and concentration-dependent. The carcinogen does not bind selectively to an identifiable genetic locus. There is an apparent preferential binding to the band regions of the chromosomes. Since most of the chromosomal mass resides in the band regions, it can be estimated that the amount of carcinogen bound per unit DNA is the same for both bands and interbands. In these chromosomes the folding pattern of the chromatin fiber does not markedly influence the binding of the carcinogen. Since the carcinogen did not bind to a defined genetic locus, it is concluded that it binds to the chromosome in a random manner. 6) Mice immunized with diol epoxide I-modified DNA produce antibodies which will specifically react with the modified DNA, but not with unmodified DNA. 7) The various procedures involved in the monoclonal antibody technique have been learned. A solid-phase enzyme-linked immunoassay specific for diol epoxide I-modified DNA has been established. 8) Analyses of 72 hybridoma cell cultures by the solid-phase enzyme-linked immunoassay detected 3 cultures which produced antibody specific for diol epoxide I-modified DNA. Further characterization of the clones and subculture of individual cells is in progress.

Significance to Biomedical Research and the Program of the Institute: Understanding the processes involved in the interaction of chemical carcinogens with the genome of a target cell may help elucidate various aspects of the mechanism of carcinogenesis. The approaches used may be generally applicable to studies on the damage to cells induced by carcinogens and its subsequent repair.

Proposed Course of Project: Studies aimed at accomplishing the goals of this project will be continued using the experimental approaches described above.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05125-01-LMC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Study of Multiple Forms of Rabbit Microsomal Cytochrome P-450 by Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Sun-Ju (Kwon) Cha	Guest Worker	LMC	NCI
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COOPERATING UNITS (if any)
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LAB/BRANCH
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SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rabbit liver microsomes provide 4 electrophoretically distinctive forms of cytochrome P-450, LM1, LM2, LM4 and LM7 molecular weight of which are 47,000, 48,700, 55,300 and 60,000 daltons respectively. LM2 is a predominant form induced by phenobarbital, LM4 predominant form induced by -naphtoflavone even all forms are detectable in uninduced rabbit microsomes as constituents of multiple forms of cytochrome P-450. The cytochrome P-450 is the enzyme responsible for mixed function oxidase (aryl hydrocarbon hydroxylase) which activates benzo[a]pyrene to ultimate carcinogen, benzo[a]pyrene-7,8-diol-9,10-epoxide with the function of epoxide hydratase. Monoclonal antibody to P-450 LM2 made by hybridoma between myeloma and mouse spleen cells derived from mice immunized with P-450 LM2 specifically binds, precipitates and inhibits the enzyme activity of P-450 LM2 but not P-450 1M1, LM4 and LM7. The purpose of this work is to investigate the ability of monoclonal antibodies to distinguish specifically the different forms of cytochrome P-450.

Project Description

Objectives: Monoclonal antibodies are produced by hybridomas of a single hybrid origin. Therefore, monoclonal antibodies contain only one kind of antibodies specific to the antigen with respect to a certain specific antigenic determinant. Four electrophoretically distinctive forms of cytochrome P-450 were found in rabbit liver microsomes, LM1, Lm2, Lm4 and LM7. LM2 and LM4 are the predominant forms induced phenobarbital and -naphtoflavone respectively. The multiple forms of P-450 are primary enzymes involved in benzo[a] pyrene metabolism leading to ultimate carcinogen, benzo[a]pyrene-7,8-diol-9,10-epoxide with the function of epoxide hydratase. Therefore, the purpose of this work is to investigate the ability of monoclonal antibody to distinguish different forms of microsomal cytochrome P-450, using rabbit microsomal P-450 as a model system.

Methods Employed: Balb/c female mice were immunized with purified rabbit cytochrome P-450 LM2 for 4 weeks intraperitoneally. The primed spleen cells were hybridized with myeloma cells, azaguanine resistant using polyethylene glycol and hybrid cells were selected in HAT medium which allowed only hybrid cells to grow. The production of monoclonal antibody was detected in culture fluids by solid radioimmunoassay and the class of immunoglobulin by Ouchterlony immunodiffusion with concentrated culture fluids and antiimmunoglobulins. Double immunodiffusion analysis was also done to test the ability of monoclonal antibodies to precipitate different forms of P-450. The tests of the effect of monoclonal antibodies to P-450 LM2 on the enzyme activities of P-450 LM1, LM2, LM4 and LM7 were carried out in the presence of the P-450 and monoclonal antibodies in reconstituted systems using benzo[a]pyrene as a substrate and measuring 3-OH-benzo [a]pyrene as the product with fluorometer. Monoclonal antibodies were also obtained as ascites of mice.

Major Findings: (1) Four out of 206 independent hybrid clones tested produced monoclonal antibodies the class of which were mouse IgG1. (2) The monoclonal antibodies specifically were able to bind and precipitate P-450 LM2 but not P-450 LM1, 1M4 or LM7. (3) The monoclonal antibodies specifically inhibited the enzyme activity of P-450 LM2 but not the activity of P-450 LM1, LM4 or LM7. (4) The two out of 4 antibody producers were stable but the two others lost their ability to produce monoclonal antibodies by subcultures. The abstract of this work has been submitted to the annual meeting of American Association for Cancer Research and two papers are in preparation.

Significance to Biomedical Research and the Program of the Institute. Metabolism of benzo[a]pyrene leads to both directions: detoxification producing water soluble conjugates and carcinogenesis processes producing the ultimate carcinogen, benzo[a]pyrene-7,8-diol-9,10-epoxide. The fate of benzo[a]pyrene metabolism might be determined by a specific form of P-450 which can be the predominant form by the preexposure of

individuals to a specific inducer such as benzy[a]pyrene. The presence of multiple forms of cytochrome P-450 in human liver microsomes also has been reported recently. Therefore, the study of multiple forms of P-450 by monoclonal antibody is important to investigate the specific and/or predominant form of P-450 for individual differences in susceptibility to carcinogens and screen for individuals prone to cancer.

Proposed Course: Explore the usefulness of monoclonal antibodies in examing various enzymes of carcinogen metabolism.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CP 05126 01- LMC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biophysical Analysis of DNA Modified by Nucleophilic Drugs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The topic of the present study is the establishment of sensitive and accurate methods for the detection of structural modifications of DNA caused by covalent and non-covalent interaction with nucleophilic drugs.

Two dimensional gel electrophoresis of DNA, under different experimental conditions provided an accurate measurement of the superhelical densities, the directions of the superhelical turns, the binding constants, and the relative enthalpies and entropies of DNA bound by intercalating drugs.

The forms of interactions were studied by a number of techniques, such as electron microscopy, NMR, spectroscopic analysis. Data obtained were analyzed by computer graphic technology to observe possible atomic configurations.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: (1) To observe the conformational changes of DNA caused by the covalent and non-covalent interactions with nucleophilic drugs; (2) To observe the biochemical and biophysical nature of the interactions between DNA and drugs; (3) To make available information obtained from basic experi-experimentation for the understanding of the structural organization of the genetic components of living organisms.

Methods Employed: The nucleophilic drugs used for the study are: poly-cyclic hydrocarbon metabolites, ethidium bromide, propidium diiodide, psoralen, amino acid pyrolysates, acinomycin D, etc.

(1) Covalently closed DNA with different superhelical densities was produced by incubating linearized pBR322 DNA (by endonuclease E. co.R1 an organic solvent. Two dimensional agarose gel electrophoresis was carried out, adding ethidium bromide only in the second electrophoretic run.

(2) The linearized pBR322 DNA was mixed with different concentrations of psoralen and irradiated with long wave ultraviolet light (365 nm). An aliquo of the DNA was brought to 90% formamide and 0.004% cytochrome c and spread over the surface of a 50% formamide solution. The DNA sample was transferred onto a colloid ion membrane coated grid for electron microscopic observation. Control DNA received either no treatment with psoralen or UV irradiation. Another aliquot of the DNA was analyzed by gel electrophoresis.

(3) An oligonucleotide with three DNA base pairs was produced by computer graphic technology. Graphic models of benzo(a)pyrene (BP) metabolites, such as 7,8-dihydrodiol BP, 7,8,9-triol BP, 7,8,9,10-tetrol P, 7,8-diol-9,10-epoxide, psoralen, and ethidium bromide were also constructed. Crystallo-graphic atomic coordination parameters, energy calculations, and physical space filling models were used for input information for computation. The interactions between the DNA and drugs were observed by a stereo projection, which appeared on a color TV screen. These were photographed and atomic and molecular configurations were analyzed.

Major Findings: The two dimensional gel electrophoresis allowed us to look into: 1) The relative mobilities fo the open circular DNA and the nearly relaxed closed circular DNA; 2) The relative mobilities of the positively and negatively supercoiled DNAs having equal numbers of suprhelical turns, and; 3) The direct determination of the superhelical density of any closed circular DNA.

By using this method, we are able to determine the number of superhelical turns induced by drug intercalation, winding angle of the DNA helix, binding constant, the enthalpy, and entropy for the intercalating angles.

Near UV irradiation of DNA in the presence of psoralen is known to cause interstrand cross linkage of DNA bases. The method developed in our laboratory was found to be highly useful for quantitation of the cross linkage between complementary strands under different experimental conditions. The

number of cross linking sites could be observed under an electron microscope and was proportional to the dose of psoralen under a given dose of UV irradiation.

(3) Insertion (intercalation) of benzo(a)pyrene metabolites with hydroxylation at 7,8 positions between DNA base pairs was found to be possible in such a way that the aromatic pyrene moiety intruded on the base pair region leaving the hydroxylated region oriented closely to the sugar-phosphate backbones of DNA. BP metabolites having hydroxylation or oxidation at positions other than 7 and 8 were found to be incapable of intercalation, suggesting that hydroxylation at the 7 and 8 positions is essential for intercalative activity to take place. There was no possibility of intercalation of diol-epoxide which was covalently bound by its carbon 10 position to the 2-amino group of guanine in double stranded DNA.

Significance to Biomedical Research and Program of the Institute: Accumulating scientific data suggest that the maintenance of the proper conformation is essential for the genetic functions of DNA, including replication and transcription.

The analysis of superhelical DNA structure, both normal and modified by drug interaction provides useful information for our understanding of its macromolecular organization and the functional aspects of the genetic machinery in living systems. Psoralen is a drug commonly used clinicly for the treatment of psoriasis. However, a potential risk, based on its nucleophilic and cross linking activity has not been entirely ruled out. The method of assaying DNA crosslinkage developed in the present study will be highly useful for elucidating the mechanism of DNA damage induced by the combined effects of the drug and UV irradiation.

Macromolecular interaction demonstrated by computer graphic techniques made it possible for us to observe the three dimensional structures of DNA and the molecular dynamics of the drugs' interacton. The system could be applied to a variety of DNA modifications produced either by biochemical or by biophyical means.

Proposed Course of the Project: Two dimensional gel electrophoresis will be used for the analysis of DNA modified by other drugs known to interact with DNA in various ways. Particular emphasis will be placed on drugs with mutagenic and carcinogenic activities and cancer chemotherapeutic agents active against cancer.

The effects of psoralen and its derivatives and UV irradiation combined on DNA will be studied further by isolating the crosslinked regions by enzymatic hydrolysis followed by HPLC analysis.

Publications

Mizusawa, H. and Kakefuda, T.: Inhibition of DNA synthesis in vitro by bindin of benzo(a)yrene metabolite diol-epoxide I to DNA. Nature 279: 75-78, 1979.

Kimura, M., Otaki, N., and Kakefuda, T: Characterization of cadmium-induced metallothioein in African green monkey kidney cells in vitro. In Kagi, J..R. and Nordberg, M. (Eds.): Meallothionein. Basel, Boston & Stuttgart, Birkhauser Verlag, 1979,

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05127 01-LMC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Development of An Improved High-Performance Liquid Chromatographic Technique of Analysis of Benzo(a)pyrene Metabolites and Applications to Carcinogenicity Problems.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	H.V. Gelboin	Chief	LMC	NCI
	F.P. Guengrich	Associate Professor of Biochem., Vanderbilt Univ., Tennessee		

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Molecular Carcinogenesis, Carcinogenesis Intramural Program

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.4	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Improvement of an existing high-performance liquid chromatographic (HPLC) technique of analysis of Benzo[a]pyrene (BP) metabolites is being pursued in this laboratory and is being applied to carcinogenesis related problems in collaborative projects to determine the relationship between metabolism and carcinogenicity of Benzo[a]pyrene. Studies involve: (1) evaluation of various commercially available HPLC chromatographic columns for separation and analysis of Benzo[a] pyrene metabolites, (2) optimization of the chromatographic method by using various solvent mixtures and solvent gradient programs, (3) identification of unknown metabolites of Benzo[a]pyrene in activation and metabolism studies by mass spectrometry and (4) applications of high-performance liquid chromatography as an analytical tool for the analysis and determination of metabolites by in vitro metabolic action of different enzyme systems on radioisotope-labelled substrates.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: (1) To develop an improved and completely (or almost completely) automated high performance-liquid chromatographic method of analysis of all (or almost all) the metabolic products of Benzo[a] pyrene. (2) To apply the high-performance chromatographic technique to analysis of metabolites in Benzo[a]pyrene activation and metabolism studies using substrates labelled with ^{14}C and ^{3}H. (3) To identify the still unknown metabolites of Benzo[a]pyrene by mass spectrometry.

Methods Employed: Several commercially available reverse-phase analytical chromatographic columns are being evaluated using spectroquality solvents for various gradient programs. The existing high-performance liquid chromatography instrument is being modified by coupling the columns with the aid of switching valves. A number of metabolites of Benzo[a]pyrene are being isolated after chromatography for mass spectral analysis.

Major Findings: Independent studies are being done to obtain high resolution of all the metabolites of Benzo[a]pyrene especially the phenolic products which have not been completely separated by existing chromatographic techniques except by one old reverse-phase column. The latter had deteriorated in efficiency but was regenerated by leaching with various organic solvents resulting to a column with a higher resolving power. In an attempt to optimize the resolution of Benzo[a] pyrene metabolites, various solvent gradient systems have been tried on several chromatographic columns and although some improvement on the separation of the phenolic metabolites have been achieved, the resolution obtained is not quite satisfactory.

In collaborative projects in vitro metabolic studies of Benzo[a]pyrene involving human P-450, high-performance liquid chromatographic analysis of the metabolites gave satisfactory separation and quantitation.

Significance to Biomedical Research and the Program of the Institute: Development of a highly efficient and automated HPLC method of separation and analysis of Benzo[a]pyrene metabolites will greatly help in the determination of the relationship between metabolic characteristics of various enzyme systems towards Benzo[a]pyrene and the carcinogenicity of the latter.

Proposed Course of Project: More HPLC columns of different selectivities will be evaluated using various solvent systems to obtain several high resolution methods of Benzo[a]pyrene metabolites. The desired methods thus obtained will be applied to in vitro metabolic products of different enzyme systems (human cells as well as purified human P-450 with and without flavone inhibitors and inducers) on ^{14}C and ^{3}H labelled Benzo[a] pyrene substrates.

Project No. Z01-CP-05127-01-LMC

Publications

Menard, R.H., Guenthner, T.M., Taburet, A.M., Kon, H., Pohl, L.R., Gillette, J.R., Gelboin, H.V. and Trager, W.F.: Specificity of the in vitro destruction of adrenal and hepatic microsomal steroid hydroxylases by thiosteroids. Molecular Pharm. 16, 997-1010.

SUMMARY REPORT

LABORATORY OF MOLECULAR VIROLOGY

October 1, 1979 through September 30, 1980

The Laboratory of Molecular Virology plans and conducts research on viruses to define their potential role in the development of cancers in humans and animals; develops and applies biological, biochemical and immunological procedures to obtain evidence for the presence of virus genetic information in cells; investigates mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells; and uses viruses as tools for probing cellular regulatory mechanisms.

The Virus Tumor Biology Section studies the mechanisms of cellular transformation induced by human and simian papovaviruses; investigates transcription and processing in infected cells; examines genetic function in terms of molecular structure; and determines the influence of cell differentiation on virus genetic expression. The Virus Tumor Biochemistry Section conducts research on specific protein and nucleic acid components of cell-transforming viruses, including the isolation and characterization of virion structural and nonstructural proteins and the cloning of DNA segments in prokaryotic vectors for studies on biologic function.

The Microbiology Section studies the biology of herpes viruses, their association with certain cancers in humans and animals with emphasis on the elucidation of the mechanisms whereby viruses of this group establish occult infections in cells which may terminate in neoplasia, their effect on normal cellular control mechanisms and the effects of their interaction with type cDNA viruses in dually infected cells. In addition to conducting their own research programs, the LMV sections participate in collaborative efforts, which include seminars, data presentations, and research projects.

A principal objective in one line of research has been the elucidation of regulatory signals responsible for generation and stabilization of mRNA molecules. Considerable progress has been made toward this goal through the use of viral mutants and recombinant DNA molecules. We have demonstrated that removing splice junctions from certain mRNA sequences renders the subsequent transcript unstable. Specific deletions of the precise SV40 16S intervening sequence, furthermore, shows that something associated with the act of splicing is critical for stabilization of the late 16S SV40 mRNA. This intron negative transcript has now been rescued by the insertion of the small βglobin^{maj} intervening sequence with its flanking sequences, but only in the orientation of the SV40 late mRNA. These experimental results indicate that while the polarity of a splice junction is critical, the source of the DNA and the position of insertions are less critical.

Recombinant molecules between SV40 as a vector, and the rat preproinsulin gene, have been constructed. Studies with these recombinants suggest that the three major regulatory signals of the rat gene can be used the promoter, the splice sites, and the polyadenylation signals. Future studies with this and similar recombinants will be directed toward an analysis of the nucleotide signals necessary for these three major events.

In a parallel set of studies, we have made use of the naturally occurring recombinant molecules formed between adenovirus and SV40 DNA. Analysis of transcripts from one of these hybrid viruses $Ad2^{+}ND_4$ del, provides evidence for the use of a new acceptor splice junction within the SV40 early region. This acceptor splice junction is of interest for at least three reasons: 1) it is not used at detectable levels in the early SV40 mRNAs, 2) it is part of a hybrid splice junction with the donor sequence arising from the adenovirus X leader, and 3) this splicing event appears to occur only late after adenovirus infection and presumably depends on the absence of the normal SV40 splice sites which are not present in the ND_4 del population.

An indepth study was made of the early SV40 mRNAs and the T-antigens which they encode. Results of these experiments suggested that the relative amount of the large T-, small t- mRNAs, and consequently their proteins, depends on the cells which were inoculated, and the temperature at which they were incubated. In addition, a 70 nucleotide small RNA molecule was identified which has the potential to bind to the early SV40 messenger RNA. The role of this small RNA is unknown, but its specificity in terms of size and site of binding suggest that it may be a control element. It certainly warrants further study.

At present, the only known cells which do not express early SV40 mRNA are mouse teratocarcinoma cells. We have shown that at least one block to this expression resides at the level of mRNA processing. Differentiation of these cells with retinoic acid, renders them susceptible to the early events of an SV40 infection with subsequent production of stable early SV40 mRNA and T-antigens.

A new DNA sequencing technique, a modification of the enzymatic method of Maat and Smith, has been developed. Among the advantages of this technique are the rapidity and reliability in obtaining sequences of interest. This method was used to derive the entire DNA sequence of human papovavirus BKV (DUN). A comparison of the BKV sequence to those already obtained in other laboratories for SV40 and polyoma virus has revealed a number of interesting analogies. In addition to putative regulatory signals, we have been able to identify the presumed protein sequences for the BKV early and late polypeptides. By comparing the putative BKV splice sites to a large number of known splicing sequences, we have been able to extend the so-called "Chambon rule" for splicing. The value of these extended rules lies in their ability to provide the prediction of splice sites from a known DNA sequence and approximate sizes of mRNA exons.

In a study directed at the potential interactions between papovaviruses and adenoviruses, we reached the conclusion that these viruses can share biologically active virus encoded polypeptides. Individual experiments revealed: 1) SV40 coinfection enables the DNA-negative adenovirus mutant ts125 capable of replication at the nonpermissive temperature. 2) Adenovirus coinfection leads to partial restoration of the regulation in the balance of early to late SV40 transcription by SV40 mutant tsA58. 3) SV40 tsA mutants, even at the nonpermissive temperature, are capable of providing helper function for adenovirus replication in monkey kidney cells. 4) Critical regions in both the adenovirus and SV40 genomes can be shown to contain limited DNA sequence homology in hybridization experiments performed under conditions of reduced stringency.

We have established an in vitro DNA replication system dependent on exogenous SV40 DNA I and T-antigen, which yields SV40 form I and form II DNA as products. It is hoped this system will not only provide an insight into the regulation of SV40 DNA replication, but also will serve as an in vitro complementation assay for the purification of SV40 T-antigen.

Recently, we have undertaken the molecular cloning of the gene for the mouse $H2K_b$ histocompatibility antigen as well as the gene encoding for transformation related P53 antigen. Both of these DNA segments are potentially valuable for unraveling regulatory signals associated with a) the major histocompatibility loci, b) a DNA region which encodes a protein found associated with a large panel of transformed cells.

A pseudotype of the replication defective Moloney murine sarcoma retrovirus (MSV; ml isolate) contains a phosphokinase activity that is precipitated with antibody to $pP60^{gag}$. The kinase activity transfers the γ phosphate from ATP to $pP60^{gag}$. Antibody to $pP60^{gag}$ also inhibits a phosphokinase activity partially purified from an MSV pseudotype by actin affinity chromatography. These experiments represent the first demonstration of a kinase activity associated with a defective gag polyprotein.

Considerable progress has been made in determining the genome structure of MSV and in understanding the molecular elements required for MSV transformation. Two MSV proviruses (ml and HT1) were cloned in phage λ from transformed mink cell genomic DNA. The isolates were compared by restriction enzyme analysis, heteroduplex and R-loop studies, direct nucleotide sequencing and biological activity in transformation. The ml and HT1 proviral DNA integrated at different sites in the mink genome but used the same site in the proviral DNA. The mlMSV provirus (5,200 base pairs (bp)) is smaller than HT1MSV (6700 bp) due to two ~800 bp deletions in the former occurring 2,500 and 4,700 bp from the 5' end of HT1MSV. The proviruses have 588 bp direct terminally repeated sequences (TRS) that are essential for efficient transformation and may be involved in proviral integration. The MSV specific src sequence is 1,200 bp and is located in the envelope (env) gene of the parental Moloney murine leukemia virus (MuLV). In direct DNA transfection assays, the cloned proviruses will transform cells and the MSV can be rescued from such transformants with MuLV helper. Specific portions of the cloned proviral genome were subcloned in plasmid pBR322 and used to identify regions of the MSV responsible for transforming activity. In addition to the 1200 bp src sequence, regions derived from the parental MuLV were necessary for efficient transformation. The two MuLV regions are the TRS and ~100 bp of the 5' portion of the env sequence preceding src.

A 15,000 bp fragment from normal Balb/c mouse genomic DNA that hybridized to a 600 bp fragment of MSV sarc was identified and cloned in phage λ (referred to as λ sarc).

Approximately 1,200 bp of the 15,000 bp Balb/c fragment is identical to the src sequence of MSV by heterduplex and restriction analyses. This region is defined as sarc. The normal cellular sequences flanking the sarc region showed no homology with the MuLV sequences in MSV, suggesting that the sarc sequence in MSV was acquired by some illigitimate recombination event.

However, non-MuLV sequences packaged in virions and reverse transcribed in an endogenous reaction, do hybridize strongly with a sequence 2000 bp from the 3' end of sarc. It is thought that these sequences are involved in the acquisition of sarc by MuLV. The normal cell sarc sequence was shown to be inactive in biological transformation transfection assays. However, when the MuLV sequences described above (TRS and 5' env) were substituted for the mouse cell sequences at the 5' end of sarc efficient transformation was obtained. These studies are the first to identify the genetic elements necessary for a normal cell sequence to acquire malignant potential.

Several features of the MSV TRS sequence show striking parallels with prokaryotic insertion sequence (IS) elements. Each TRS has an 11 bp inverted repeat, contains putative transcription promotor and termination signals, and has the same four bases of host sequence bracketing the integrated provirus. Moreover, a high frequency deletion event occurs between TRS elements during growth of λMSV recombinants in recombination deficient E. coli. This deletion is analogous to that observed for a prokaryotic transposition element (Tn9) that has a similar sequence organization to the integrated MSV provirus. These parallels indicate that retroviruses may have transposition like properties in eukaryotics and have structural features that are recognized in E. coli.

Bacteriophage lambda continues to serve as a paradigm for viral integration. A new region involved in the cis-regulation of integrase expression has been discovered to the left of the phage attachment site. The mechanism for this cII/cIII gene independent integrase expression is being investigated. A mutation in phage λ that enables excision in the absence of an excisionase product and the normally required host-factors has been isolated and studied. These mutants integrate more efficiently than wild type lambda into secondary attachment sites and also promote high frequency deletion formation in the phage. Frequently used secondary attachment sites recognized in normal λ have been identified by blot-hybridization. Comparison of their sequences to the primary attachment site sequence will be used to understand the nature of site specificity in viral integration. The lambda secondary attachment site in the proline operon has been sequenced. Novel attachment site mutations called site-affinity mutations are generated when lambda excises from one of two rare insertions into the galactose operon. These mutants acquire some of the bacterial attachment site sequences during prophage excision. Such mutants provide tools for studying the mechanism of attachment site recognition by integration enzymes and will give insight into the question of how DNA sequences evolve to become attachment sites for viral integration.

The heterogeneity of herpes simplex virus type 1 (HSV-1) defective DNA (dDNA) EcoR1 fragments has been clarified by examining individual molecules cloned in phage λ. Detailed physical maps have revealed that two size classes, a major (8-9,500 bp) and minor (6-8,000 bp) comprising 80 and 20% of the total dDNA can be divided into three structural groups. All of the total dDNA can be divided into three structural groups. All groups contained restriction sites characteristic of the small unique region (Us) terminal repeat sequence (TRs/IRec). Only major class Group I dDNA molecules have been previously described and contain Us sequences in the prototype orientation. All of the minor class of this group contained an apparent site specific deletion in TRs.

Group II dDNA contained Us sequences from the opposite Us permutation while Group III dDNA contains HSV sequences from non-contiguous portions of the wild type genome. This latter group is intriguing because their novel structure may indicate that the TRs sequences of HSV may be involved in genetic rearrangements.

The availability of well-characterized HSV fragments have allowed rapid progress to be made in the characterization of the IE mRNA transcripts of HSV-1 and provided for the first time, evidence for HSV-1 RNA splicing. Two IE mNRA species that are initiated within TRs or IRs terminate at opposite ends of Us and in the Us region. Translation of these messages may produce products with common N-terminal and unique C-terminal peptides. New abundant species of IE-RNA are detected in cells infected with HSV-1 containing 50% defective particles. The IE RNA is derived from the major Group I dDNA, but does not appear to serve as mRNA in cell-free translation.

The hot spot for TRs recombination in Group III dDNA would occur at the sequences adjacent to Us. A Us fragment containing this portion of TRs cloned in phage λ was shown to be extremely unstable. This recombinant had a deletion frequency 10^3 - 10^4 fold greater than comparably sized HSV hybrid phage recombinants. More than 90% of the 29 different deletions analyzed have one end point at or near the position of TRec in this fragment. This system provides an excellent means for generating deletions in the HSV Us region. These deletions have already been useful for restriction mapping Us as well as locating the immediate early (IE) mRNA transcripts from TRec. However, the most striking phenomenon with this fragment (as well as the phage cloned MSV fragments) is the prokaryotic recognition of unusual structural features of eukaryotic viruses. This may turn out to be an added bonus to prokaryotic cloning experiments and may facilitate an understanding from these sequence functions in eukaryotic systems.

Finally, a major effort has been devoted to an investigation of the potential role of HSV as a co-carcinogen in cell transformation. Evidence has been obtained to suggest that the expression of endogenous C-type viruses is altered in HSV-transformed cells. Using TK^- cells, data have been obtained which lead to the conclusion that biochemical (TK) and morphological transformation by HSV are separate events.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04876-08-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Oncogenic Virus Influence on the Biochemical Events of Host Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION
Virus Tumor Biochemistry Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Succinylacetone (SA) is a potent specific inhibitor of heme biosynthesis binding irreversibly to δ-aminolevulinic acid dehydrase, the second enzyme of the pathway. When murine erythroleukemia (MEL) cells were grown in defined medium containing SA, cellular heme concentrations decreased markedly and growth was inhibited. Growth inhibition was partially reversed when hematin was added with SA on day 0, but no reversal of growth inhibition was seen when hematin was added on day 4. The decrease of cell heme concentration was accompanied by a decrease of protein synthesis, which was partially reversed by addition of hematin to the medium. When hematin was added to the culture medium of cells which had been depleted of heme by the effect of SA, the intracellular heme levels returned to normal. Growth inhibition produced by SA was also reversed by adding hematin in the presence of SA. When heme-depleted cells were resuspended in fresh medium without SA, but containing hematin, normal levels of heme were attained within 2

hrs. The rate of heme uptake in cells incubated at both 20° and 37° was essentially the same. Both heme-depleted and cells with normal levels of heme showed uptake of hematin. SA inhibited L1210 tumor cell growth in vitro and increased the survival time of rats infected with Walker 256 ascites tumors.

PROJECT DESCRIPTION

Objectives:

To investigate the mechanism of action of SA on the growth of various tumor and normal cells in tissue culture and in rats. To study the specificity of the inhibitory activity of SA on the heme pathway and to follow the consequences of heme synthesis inhibition on other dependent biological processes in tumor cells and in rats. To study the role of endogenous and exogenous carbonyl compounds on the process of erythroid differentiation in malignant murine erythroleukemia (MEL) cells.

Methods Employed:

Conventional biochemical procedures were utilized to follow alterations in heme and protein synthesis and energy metabolism occurring in MEL cells.

Major Findings:

1. Effect of SA on heme uptake and growth of malignant MEL cells. Four, 6-dioxoheptanoic acid (succinylacetone, SA) has been shown to be a potent inhibitor of heme biosynthesis and growth in murine erythroleukemia (MEL) cells in vitro. The effect of exogenous hematin on the growth and heme levels of MEL cells depleted of heme by growth in heme-free culture medium containing SA was studied. When MEL cells were grown in defined medium containing SA, cellular heme concentrations decreased markedly and growth was inhibited. Growth inhibition was partially reversed when hematin was added with SA on day 0, but no reversal of growth inhibition was seen when hematin was added on day 4. Cellular heme levels and growth rates indicate that exogenously added hematin was taken up from defined medium. The decrease of cellular heme concentration was accompanied by a decrease of protein synthesis, which was partially reversed by addition of hematin to the medium. Concommitantly, there was a decrease of NADH-stimulated oxygen consumption in heme-depleted cells suggesting diminished respiratory reserve in these cells. When hematin was added to the culture medium of cells which had been depleted of heme by SA, the intracellular heme levels returned to normal and the growth inhibition was reversed. Following the reduction of heme levels in MEL cells by incubation with SA in heme-free medium for 4 days, these cells were resuspended in fresh medium without SA, but containing hematin. Addition of hematin was followed within 2 hrs by the return to normal cellular heme levels. Growth resumed 24 hrs after normal heme levels were restored. When the medium from heme-depleted cells was replaced by medium containing neither SA nor hematin, cellular heme levels rose slowly to the normal level at 24 hrs. This heme level was sufficient to permit growth to resume.

2. Effect of SA on L1210 cells in vitro and in rats with ascites Walker 256 tumors. L1210 cells were adapted to growth in 1-2% fetal calf serum (FCS) to decrease the possible influence of the hemoglobin present in FCS to reverse the effect of heme depletion by SA. Contrary to the situation observed with MEL cells, L1210 cells in culture did not exhibit a decrease in heme concentration after incubation with SA, but often showed increases compared to nontreated controls. At these low FCS concentrations SA at 1 mM concentrations begins to kill the cells after one doubling cycle of the cells. The marked uptake of exogenous hematin observed with MEL cells was not apparent with L1210 cells grown in medium containing low levels of FCS. SA administered via implanted osmotic pumps in rats infected with Walker 256 ascites cells was able to increase the survival time of rats by 13 to 22 days compared to rats receiving Walker tumor only.

3. Induction of erythroid differentiation of MEL cells by carbonyl compounds or their precursors. Since two inorganic selenium compounds were shown to trigger erythroid differention in MEL cells possibly by oxidizing endogenous membrane carbonyls into dicarbonyls, it is likely that certain exogenous aliphatic carbonyl compounds may likewise initiate differentiation of these cells. Hemoglobin synthesis was induced in MEL cells by 1-5 carbon compounds of various oxidation states with alcohol compounds showing less potent induction than acids, aldehydes and ketones. Aldehydes were more potent inducers than ketones.

Significance to Biomedical Research and the Program of the Institute:

Succinylacetone, as a potent inhibitor of the heme pathway, offers a different approach to chemotherapy directed against various tumor cells. The agent affects a key biochemical pathway of all cells and can ultimately cause the death of a cancer cell by disrupting its heme production, and thereupon affecting protein synthesis and respiration. The agent will also be useful for basic biochemical studies of heme synthesis, protein synthesis, and respiration. Since porphyrin uptake is a selective property of tumor cells, certain potent chemotherapeutic agents which pass through membranes with difficulty might be attached to porphyrins to facilitate their passage into tumors and cancer cells.

Proposed Course:

The effects of large doses of succinylacetone on rats and liver cells will be completed. The mechanism of action of the drug on L1210 cells in vitro will be further explored, since the biochemical responses in this cell appear to be different from those in MEL cells. The mechanism of heme uptake into tumor cells will be further characterized with the possibility that certain chemotherapeutic agents could be attached to the porphyrin molecule to enhance their uptake into tumor tissue.

Publications:

Ebert, P.S., Hess, R.A., Frykholm, B.C. and Tschudy, D.P.: Succinylacetone, a potent inhibitor of heme biosynthesis: Effects on cell growth, heme content

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Malinin, G.I. and Ebert, P.S.: Induction of erythroid differentiation in murine erythroleukemia cells by short chain aliphatic carbonyl compounds and their corresponding precursors. Evidence for a common inducing signal. FEBS Letters 109:264-266, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04882-06-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Events in Tumor Virus Replication and Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. A 15 kilobase pair (kb) EcoR1 DNA frament from normal Balb/c mouse genomic DNA containing sequences (sarc) homologous to the acquired cell sequences (src) of Moloney sarcoma virus (MSV) was cloned in phage λ. The sarc region (1.2 to 1.3 kb) of the 15.0 kb cell fragment was indistinguishable from the src region of two isolates of MSV as judged by heteroduplex and restriction endonuclease analyses. The cellular sequences flanking sarc showed no homology to MSV sequences of Moloney murine leukemia virus (MMuLV) origin (T.G. Wood, project #Z01-CP-05156-01-LMV). Cloned subgenomic portions of MSV that contain src transform NIH-3T3 cells in vitro with high efficiency (Z01-CP-05103-01-LMV). In this same assay, the cloned normal cell sarc fragment was inactive. A hybrid DNA molecule, formed by introducing M-MuLV sequences 5' to sarc activated the transforming potential of sarc. The biological activity of this hybrid molecule, coupled with determinations of the biological activity of cloned portions of the MSV genome demonstrated that two regions of the provirus

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derived from M-MuLV, are required for efficient transformation by src/sarc.

2. New classes of immediate early (IE) mRNAs have been detected in cells infected by defective particles containing herpes simplex virus type 1 (HSV-1) stocks. These IE mRNA species are apparently transcribed from defective DNA molecules present in the virus population. They do not serve as mRNAs in a cell-free translation system. Two of the IE mRNA of wild type HSV-1 which originate at the same locus within the terminal and internal redundant sequences (TRs/IRs) of the HSV-1 genome terminate in different sequences within the unique S region (Us) of the genome. Both 2kb mRNA species have a common 260 base 5' terminal sequence mapping in TRS. This common sequence is spliced to different mRNA bodies coded for mainly by Us sequences.

PROJECT DESCRIPTION

Objectives:

To investigate biochemical events associated with tumor virus replication and to determine their relationship to neoplastic transformation of host cells.

Methods Employed:

Oncogenic viruses are propagated in tissue culture. Focus assays and plaque assays are used for the detection of some propagated and purified viruses. Other assays are: agarose gel diffusion for the detection of viral antigens; polyacrylamide gel electrophoresis and guanidine-agarose chromatography for the purification and identification of virus-specific proteins; in vivo and in vitro labeling of purified viruses using radio-labeled amino acids and nucleic acids. Sensitive peptide mapping techniques are developed for use in comparing virion polypeptides. Endonuclease restriction enzyme cleaveage of viral and cellular DNA. Cloning in bacterial and mammalian vectors of specific virus related sequences of viral and host cell origin.

Major Findings:

I. We previously cloned and characterized the integrated proviruses of two MSV isolates, ml and HT1, into phage lambda. In each provirus the src portion as well as sequences derived from M-MuLV were identified. The src regions of both ml and HT1 MSV are identical by heteroduplex and restriction endonuclease analyses. Normal mouse cellular sarc sequences were detected in Southern blots of genomic EcoR1 digested DNA using a 700 bp nick translated src specific fragment from λ HT1 as probe. A single hybridizing region, 15kb, was enriched and cloned into phage λ. Heteroduplexes formed between this clone and the recombinant phages λml or λ HT1 showed only one region of homology which corresponded to the src region of both MSV proviruses. This region, ~1.2-2.3kb in length, occurs 3kb from one end and 11kb from the other end of the 15kb cell sarc fragment. At this level of resolution, both src and sarc appeared homologous and colinear. A comparison of endnuclease restriction cleavage

maps of the MSV src and sarc regions showed both to possess the same series of nine restriction sites. However, other than the sarc and src regions there were no similarities between the remaining ~14kb of the cell fragment and either of the MSV proviruses. In addition, no M-MuLV sequences were detected in the cell sarc fragment by Southern transfer using a nick translated cloned M-MuLV probe. However, an M-MuLV complementary DNA (c-DNA) probe hybridized strongly to a 4kb region 2kb from the 3' end of sarc sequence. This sequence is not related to M-MuLV, but is packaged in virions and is reverse transcribed in the endogenous c-DNA reaction (see T.G. Wood #Z01-CP-05156-01-LMV). The presence of this sequence in M-MuLV could be related to the generation of MSV from M-MuLV.

The cloned integrated proviral DNA fragments of ml and HT1 MSV produce foci of transformation on NIH 3T3 cells in a direct DNA transfection assay. The normal mouse sarc sequence was inactive in this assay. We utilized common restriction sites in the 5' and 3' termini of src and sarc as loci for generating hybrid leuk-sarc molecules between sarc and the complementary 5' and 3' segments of MSV adjacent to src. Only a hybrid with MSV sequences substituted to the 5' end of sarc was active in the biological assay. These results, coupled with the transfection-transformation studies with subgenomic portions of MSV (W. McClements #Z01-CP-05103-02-LMV) indicate that two M-MuLV regions of the MSV genome are required in addition to src/sarc for efficient transformation. One M-MuLV region is the 600 base pair terminal repeat sequence of the provirus. The presence of this sequence either 5' to 3' to an internal MSV src sequence enhances transforming activity 1000-fold. The other MuLV region represents approximately 100 nucleotides preceeding the 5' end of the src sequence. The requirement of this region for efficient transformation is deduced by i) comparison of the endonuclease restriction maps of three MSV provirus isolates, ii) eliminating specific MuLV sequences in cloned subgenomic fragments without loss of biological activity, and iii) demonstrating that efficient transforming activity of the cell sarc sequence occurs only when MSV sequences have been substituted to replace the normal cell sequences at its 5' end.

II. The five predominant immediate early (IE) mRNAs of herpes simplex virus type 1 (HSV-1) are synthesized in cells infected with HSV-1 in the continuous presence of the protein synthesis inhibitor cycloheximide and comprise the earliest temporal class of virus transcripts. Three of the IE mRNAs, designated IE mRNA-3 through 5, map in regions of the virus genome which are present in defective DNA molecules of the Patton strain of HSV-1. Consequently, we have used the Northern blotting technique to compare the IE mRNAs made in cells infected with wild-type virus (P0), to those made in cells infected with a virus population (P13), containing defective particles. In these analyses, polyadenylated cytoplasmic IE RNAs were separated by electrophoresis through denaturing agarose gels, and the transcripts were transferred to activated cellulose paper by blotting. The IE RNAs bound to paper were identified by hybridization with defined ^{32}P-labeled HSV-1 DNA probes. The synthesis of a number of novel P13 IE RNAs was observed, indicating that defective DNA molecules which contain novel sequence arrangements, were transcribed. These novel P13 IE RNAs did not serve as mRNAs in a cell-free translation system.

Recent studies have involved analyses of the fine structure of certain IE mRNAs. The techniques utilized were the Berk and Sharp mapping technique and R-loop analysis. In the first method, the regions of specific HSV-1 DNA fragments protected against single-strand specific nuclease digestion by duplex formation with complementary cytoplasmic IE RNA sequences, were analysed by gel electrophoresis. In the R-loop analyses, hybrids formed between HSV-1 DNA fragments and ID mRNAs were mapped by electron microscopy. These studies indicated that IE mRNA-4 and IE mRNA-5 contain a common 260 base 5' terminal sequence mapping in the inverted reiterated region (TRs/IRs) of the S component of the virus DNA. This sequence is spliced to different cotranscripts containing sequences mapping partially in TRs/IRs and partially at opposite ends of the S unique (Us) region. These studies provided the first evidence of splicing of HSV-1 transcripts. Further, they suggest that HSV-1 may use this unusual mechanism to generate mRNAs whose translation products contain common N-terminal peptides and unique C-terminal peptides.

Significance to Biomedical Research and the Program of the Institute:

The cloning of tumor virus genomic and sub-genomic fragments in bacterial vectors represents a major advance facilitating the study of virus-induced transformation. Not only is it now possible to obtain unlimited quantities of genomic nucleic acid where previous investigations were hampered by infinitesimally small amounts, but as we have demonstrated, it is now possible to identify and isolate by recombinant DNA cloning techniques, a single set of nucleotide sequences responsible for the transformation of cells from sequences representing the entire cellular chromosome. We can now precisely determine the portion of the viral genome responsible for cellular transformation and determine its primary sequence. The MSV src/sarc experiments identify for the first time, specific genetic elements that are required for a normal cell sequence to acquire malignant potential. Chromosomal rearrangements of genetic elements with similar phenotypes could presumably induce nonviral mediated oncogenesis.

Proposed Course:

1. Specific viral and cellular sequences responsible for malignant transformation will be identified and characterized.

2. The early events in herpes simplex virus replication will be characterized at the molecular level.

Publications:

Vande Woude, G.F., Oskarsson, M.K., Enquist, L.W., Nomura, S., Sullivan, M. and Fischinger, P.J.: The cloning of integrated Moloney sarcoma proviral DNA sequences in bacteriophage lambda. Proc. Natl. Acad. Sci. USA, 4464-4468, 1979.

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Sherr, C.J., Fedele, L.A., Oskarsson, M., Maizel, J.V. and Vande Woude, G.F.: Molecular Cloning of Snyder-Theilen Feline leukemia and sarcoma viruses, J. Virol., in press.

Blair, D.G., McClements, W.L., Oskarsson, M.A., Fischinger, P.J. and Vande Woude, G.F.: Biological activity of cloned moloney murine sarcoma virus DNA-Evidence that terminally redundant DNA sequences enhance transformation efficiency. Proc. Natl. Acad. Sci. USA, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04895-08-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of Polypeptides of Oncogenic Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Virus Tumor Biochemistry Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two independent cell lines established from mlMSV (BMuX) induced fibrosarcomas in Japanese quail showed changes in the size and cleavage stability of the mlMSV specific $pP60^{gag}$ polyprotein. The size of the gag polyprotein in these cell lines was 2000 and 6000 daltons less than $pP60^{gag}$. The change in molecular size of the new polyproteins was not altered in infected rat cells which indicated genetic alterations in the mlMSV genomes. A phosphokinase was isolated from MSV (MMuLV) pseudotype virions by actin affinity chromatography that interacted with antibodies to $pP60^{gag}$. This demonstrated an immunological interaction between a sarcoma specific polypeptide and a cytoskeletal polypeptide.

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(Rev. 10-76)

PROJECT DESCRIPTION

Objectives:

To develop biochemical and immunological methods; to identify, isolate and characterize transformation specific viral polypeptides. Particular attention is given to the immunological detection and biochemical characterization of polypeptides associated with transformation. These methods will serve as tools for examining the molecular events associated with both viral replication and transformation.

Methods Employed:

Viruses were propagated in cell culture and in Japanese quail. Focus-forming assays, virus neutralization, immunofluorescence and immunoprecipitation methods were employed to determine the presence, extent, and type of virus infection. Specific antibodies to purified virus polypeptides prepared in goats, rabbits, and quail and were used to immunoprecipitate cell cytoplasms. Immuno precipitates were analyzed by electrophoresis and autoradiography.

Major Findings:

1. We have previously described the characteristics of the mlMSV specific gag polyprotein, pP60^{gag}. Briefly, P60 was shown to be a kinetically stable polyprotein consisting of two mMuLV structural proteins, p12 and p15, and part of a third structural protein, p30. The carboxyl terminus of P60 (approximately 5,000 daltons) was determined to be mlMSV specific in that it was not related structurally to any of the known leukemia virus structural proteins. Antigenic determinants of p12, p15, and p30 were conserved in P60. A variety of mammalian cell lines (mouse, cat, rat, dog, mink, and human) have been infected with mlMSV and in each case, the molecular size and stability of P60 remained unchanged. Furthermore, when the complete genome of mlMSV was cloned in a lambda bacteriphage vector and transfected back into a mink cell line, the newly transformed mink cell synthesized a P60 indistinguishable from that specified by the parental mlMSV. Thus, the genetic formation of mlMSV appeared to be quite stable. We constructed an appropriate pseudotype of MSV, mlMSV (BMuX), to study the replication of this MSV in avian cells. Quail embryo cells infected with this mlMSV (BMuX) when inoculated into Japanese quail, produced fibrosarcomas in 80% of the test birds. Cell cultures were established from several of these tumors and the replication of MSV was examined. In two such cell cultures, QT12 and QT106, derived from two independent preparations of quail embryo cells, we determined that the P60 equivalent for QT12 was p58 and in QT106, p54. Also, the kinetic stability of the new MSV specific polyprotein was markedly reduced in quail cells. Moreover, QT12 and QT106 cells, were transplanted into other Japanese quail and additional tumor derived cell cultures, (QT103 and QT106-41 respectively) were examined for the presence of MSV-specific polyproteins; the characteristic QT12 p58 and QT106 p54 was detected. These analyses could not exclude that smaller gag product was due to the processing of MSV in avian cells. Therefore, MSV in QT12 and QT106 were rescued and infected back into rat cells. Even in these cells, the MSV-specific polyproteins p58 from QT12 cells and p54 from QT106 cells

was observed. This indicated that a unique alteration of the mlMSV genome occurred during MSV infection into avian cells or during tumor formation in birds. It is of interest that the two independently established tumor lines gave populations of cells in culture, with essentially a monoclonal variant of MSV. It may be that portions of mlMSV P60 promote tumor rejection in Japanese quail and only when this region is deleted can tumor formation proceed. In any case, a genetically stable variation, as reflected by the alteration of a MSV-specific protein, has not previously been observed in mammalian cells.

2. The temperature sensitive mutant of MSV 124 was used in experiments to study synthesis of MSV specific proteins. The stable group antigen gene (gag) precursor protein for this isolate was determined to have a molecular weight of 58,000 daltons and was shown to be synthesized and phosphorylated at the transformation permissive (34°C) and nonpermissive (39°C) temperatures. The p58 contained p12, p15 and p30 antigenic determinants which was characteristic of the stable gag precursor polyproteins previously described. Two additional proteins were partially characterized in this study. A protein with a molecular weight of 85,000 daltons was detected in cells grown at the permissive temperature, but not detected at the nonpermissive temperature. This p85 contained p12, p15, and p30 antigenic determinants. The non-gag components of this molecule are as yet undetermined. The second protein characterized in this study was a thermolabile protein kinase isolated from homologous pseudotypes of the temperature sensitive MSV. The kinase was isolated from virons by actin affinity chromatography. The enzyme was shown to be MSV specific and thermolabile at the nonpermissive temperature. Further, this kinase interacts with antibodies to the stable gag precursor P60, of mlMSV. This interaction could not be altered by pretreatment of anti-P60 serum with structural proteins of MMuLV. This data demonstrated an immunological interaction between a cytoskeletal protein and a MSV specific protein, P60, perhaps through the carboxylterminus of P60, since this region (approximately 5,000 daltons) has been shown not to be related to the parental MMuLV gag region polyeptides.

Significance of Biomedical Research and the Program of the Institute:

One cloned MSV, mlMSV, was previously shown to specify an uncleaved stable precursor, $P60^{gag}$, to the major oncornavirus structural proteins in transformed mammalian cells. When this same MSV was used to transform avian cells, the MSV polyprotein marker was found to have a lower molecular weight and was no longer stable in kinetic experiments. This modification event appeared to happen during tumor formation and once the modification occured it became genetically stable. This observation has provided a unique model for studying retrovirus transformation in avian cells.

Proposed Course:

Immunological and biochemical techniques will be used to determine the events leading to the modification of mlMSV in avian cells. This approach, in addition to what is known about the other related defective Moloney sarcoma viruses, may expand understanding of the model(s) proposed for the generation

of defective sarcoma viruses from competent leukemia viruses. We now have available two altered MSV genomes that will be examined by restriction endonucleases methods to determine the exact location of the probable recombination.

Publications:

Sen, A., Todaro, G.J., Blair, D.G., and Robey, W.G.: Thermolabile protein kinase molecules in a temperature sensitive murine sarcoma virus pseudotype. Proc. Natl. Acad. Sci. USA, 76:3617-3621, 1979.

Wood, T.G., Horn, J.P., Robey, W.G., Blair, D.G. and Arlinghaus, R.B.: Characterization of viral specified proteins present in NRK cells infected with a temperature-sensitive transformation mutant of Moloney Murine Sarcoma Virus. Cold Spring Harbor Symposia on Quantitative Biology, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04902-08-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

In vivo and in vitro Translation of Tumor Virus Specific Polypeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Ascione	Research Chemist	LMV NCI
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	W.G. Robey	Research Chemist	LMV NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have isolated immediate early (IE) viral messenger (m) RNA from vero cells infected with Herpes Simplex Virus (HSV) in the presence of cycloheximide (cx), a mRNA fractionated by density gradient sedimentation of enriched by oligo dT-cellulose chromatography was translated. IE mRNA cell free and specific polypeptide products were identified. Specifically, we obtain proteins in a cell free system of 175,000, 110,000 and 68,000 daltons which are characteristic of IE HSV specific products seen with cells labeled in vivo. We have similarly obtained from defective HSV infected vero cells a species of IE mRNA and have compared products in a cell free translation to the nondefective HSV mRNA specific polypeptides. Both mRNAs specify the polypeptide products of 175,000, 110,000 and 68,000 daltons, however the levels synthesized of 175,000 dalton product using a comparable quantity of mRNA is 2-3-fold greater with defective mRNA, Vmw 135 seen in the non-defective IE mRNA cell free translation system was markedly diminished in the defective IE mRNA translation.

In studies using mRNA from HSV infected cells not treated with cx, the defective (d) mRNA was found to contain fewer messages specifying virion capsid polypeptides; specifically noticeable was the paucity of a major 155,000 dalton species which was abundantly synthesized in vitro by mRNA obtained from nondefective (nd) HSV infected cells.

A mlMSV associated ATP-phosphotransferase activity was characterized; this activity was precipitated with $_{\alpha}P60^{gag}$ and was associated with virion core structures after detergent treatment and sucrose gradient fractionation.

PROJECT DESCRIPTION

Objectives:

1. To determine the translation products HSV IE mRNAs using hybrid-arrest translation assay.

2. To characterize and identify the MSV-associated protein kinase and to determine the products specified by specific mRNAs.

Methods Employed:

Guanidine extraction of whole cellular RNA from infected cells. Fractionation of RNA by oligo dT column chromatography. Preparation of rabbit reticulocytes for mRNA dependent cell-free protein synthesizing system. Cell-free translation by lysates using specific mRNA and analysis of synthesized polypeptides by linear-gradient-polyacrylamide gel electrophoresis (PAGE) and 2 dimensional analytical PAGE employing isoelectric focusing in the first dimension. Formation of specific hybrids with isolated mRNA using specific restriction fragments of HSV defective and nondefective DNAs. Analysis of ATP-phosphokinase activity using cell-free reactions with purified isopycnically banded virons and virions disrupted by detergent treatment; followed by immunoprecipitation using specific antisera directed against a viral core polypeptide (P60).

Major Findings:

1A. Immediate early mRNA isolated from defective (d) and nondefective (nd) HSV infected cells stimulates the synthesis of high molecular weight polypeptides. The predominant proteins synthesized in a cell free translation system were 175,000, 135,000, 110,000 and 68,000 daltons in molecular weight. No major qualitative differences were noted between products specific by mRNA isolated from cells infected with nd or d HSV. However, there were quantitative differences in the amounts of 175,000 and 110,00 dalton products. Specifically, these polypeptides were 2-3 fold more abundant using the d-HSV specific mRNA, while the 135,000 dalton polypeptide was very much reduced in this system when compared to that translating the nd-HSV specific mRNA.

B. The extracted d-HSV specific mRNA isolated in the absence of cx specified in a cell-free system an over-abundance of a 20,000 dalton polypeptide relative to the nd-HSV mRNA. Since other mRNA are able to be transcribed

in the absence of cx, this 20,000 dalton polypeptide may be a product specified by "early" and/or "late" HSV mRNA rather than immediate-early HSV mRNA.

C. HSV specific proteins synthesized in vivo, in the presence of cx, preferentially bind to DNA-cellulose. Similarly preparations of HSV IE mRNA stimulated polypeptides, in a cell free system were found to bind as much as 75% of this total de novo synthesized products to calf thymus DNA-cellulose.

2A. Purified detergent disrupted ml-MSV(FelV) virions contained an ATP-phosphotransferase activity. This activity was immunoprecipitated by antisera prepared against the ml-MSV specific. 60,000 dalton defective gag polyprotein (P60). Further antisera prepared against the purified gag protein p30 of RLV, as well as antisera against tween-ether disrupted Moloney-MuLV, could also precipitate this enzyme activity. By contrast, antisera prepared against murine reverse transcriptase (Ab-pol) or murine glycoprotein (Ab-gp71/69), or normal rabbit sera does not bring down the phosphotransferase activity.

B. By two dimensional polyacrylamide electrophoresis the immune precipitated virion complex ws seen to transfer P^{32} from ATP to P60. The kinased labeled P60 also coelectrophoresed with S^{35}-methionyl- and P^{32}-phosphorylated P60-labeled in vivo.

C. The virion associated phosphokinase activity both cosediments with P60 on a sucrose density gradient as well as coelutes with P60 from an affinit column of poly rA-agarose.

D. Intracellular P60 in mlMSV transformed cells was found associated with the plasma membrane. This cell fraction had also the highest levels of ATPphosphotransferase activity.

These results suggest that there may be a phosphokinase activity associated with the mlMSV specific P60, both in the virion as well as within the transformed cell.

Significance of Biomedical Research and the Program of the Institute:

These studies should serve to map the Herpes Virus specific product(s) and relate their corresponding mRNA to specific regions of the HSV genome.

The studies on the virion associated phosphokinase activity can characterize the possible biological role to its role in cellular transformation or possibl a cell regulatory function concerned with viral replication.

Proposed Course:

HSV specific mRNA will be isolated using mg quantities of cloned restricted viral DNA segments coupled to cellulose. Such mRNA can then be translated in messenger RNA dependent lysates to determine what protein(s) may be encoded in the specific regions. Further analysis by hybridization of mRNA to specifi

DNA fragment would also confirm which polypeptides are inhibited using specific HSV-DNA fragments.

These techniques are applicable to the mRNA isolated from MSV infected cells. Such mRNA isolatable by hybridizing to specific cloned DNA fragments containing sarcoma information would yield specific mRNA in quantities which could be translated in a cell free system. Such products isolated and identified could be correlated to transformation process. Sarc specific proteins could also be tested in hybrid-arrest asssays to correlate with their genomic origin.

Publications:

Ascione, R.A., Robey, W.G., Haapala, D.K.: Site of Synthesis and Intracellular Location of mlMSV Polyprotein P60. J. Gen. Virol. (1980), in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04907-04-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

DNA Sequence of Control Regions of BK Virus (BKV)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ravi Dhar	Visiting Scientist	LMV NCI
OTHER:	G. Khoury	Acting Chief, LMV	LMV NCI
	I. Seif	Visiting Fellow	LMV NCI
	C.-J. Lai	Visiting Scientist	LID NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The compelete DNA sequence of human papovavirus BKV(Dun), consisting of 5153 nucleotide pairs, was obtained. The segments of the genome which correspond to the replication origin, the tandem repeated sequences, the 5' and 3' ends of the mRNAs, the splice sites, the early and late viral proteins and the putative viral polypeptides, have been identified and mapped. The BKV DNA sequence was also compared with analogous regions in the SV40 and polyoma virus genomes in an attempt to localize viral functions for lytic growth and transformation.

2. A rapid technique was developed to sequence double stranded DNA fragments.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives:

Aims are (1) the demonstration of BKV nucleotide sequences that may be involved in the splicing of RNAs and in polyadenylation; (2) the definition of amino acids of the large T-antigen which are involved in the initiation and maintenance of transformation, viral DNA synthesis, induction of cellular DNA synthesis and the helper function for adenovirus growth in monkey cells.

Methods Employed:

The 5' end labeling of double-stranded restriction endonuclease fragments was performed using ^{32}P ATP and T4 polynucleotide kinase. The 5' labeled fragments were further digested with appropriate restriction endonucleases to generate fragments with only one labeled 5' end. These fragments were purified by polyacrylamide gel electrophoresis and sequenced by two methods: 1) the chemical degradation technique and 2) the enzymatic method with modifications.

Major Findings:

BK virus, a human virus, is known to be closely related in evolution to SV40, a monkey virus. In order to be able to correlate the nucleotide sequences of the virus to its function, we have sequenced the entire BK genome, which is 5153 nucleotides long. The origin of DNA replication, 5' and 3' ends of cytoplasmic mRNA and the N' and C' ends of the known early and late proteins have been localized. These sequences have been compared to similar sets of nucleotides from SV40 and polyoma. Using this approach and the information available to us from SV40 deletion mutants, we have been able for example, to narrow the helper function (for the growth of adenovirus in monkey cells) to less than 25 amino acids at the carboxyl terminus of T-antigen.

To precisely localize the excision points involved in splicing and maturation of BKV mRNA, we have conducted a preliminary analysis of numerous viral and eukaryotic splice site sequences. This analysis suggests that the preferred sequence for the donor site belongs to at least one of four groups: Pu↓GTAxG, Pu↓GTAxxT, Pu↓GTxxGT, Pu↓GTxAG (↓ = the cleavage site). These four groups derive from the two basic sequences, Pu↓GTxxG and Pu↓GTA. An optimal donor site might be: AG↓GTAAGT. The preferred sequence for the acceptor site is of the form PyPyxPyAG↓; no dinucleotide AG occurs within 13 nucleotides prior to the terminal AG of the 3' end of the intervening sequences. As we have found in the study of BKV splice sites, the sequences for the preferred donor and acceptor sites provide predictive value in localizing RNA splice points when the DNA sequence of the eukaryotic genes are known.

We have adapted a rapid sequencing technique from the enzymatic nick-translation method of Maat and Smith. The Forward-Backward procedure employs both synthetic and 3' to 5' exonucleolytic activities of E. coli DNA polymerase I to achieve greater reliability, especially in reading stretches of the same nucleotide. The technique has been employed to determine sequence alterations in four early SV40 temperature-sensitive (tsA) point mutants

and four SV40 viable deletion mutants. This technique was also used to determine most of the sequence of the BKV genome.

Significance to Biomedical Research and the Program of the Institute:

A comparison of the primary sequence among different papovaviruses has already provided useful information (e.g. the amino acids which are involved in the helper function for adenovirus growth in monkey cells). We hope with the use of deletion and point mutations within the early region, to better define the
amino acids within the large T-antigen which are involved in transformation, viral DNA synthesis, and induction of cellular DNA synthesis and host range.

Proposed Course:

To study the sequence of more deletions in the splicing region, and generate mutants in regulatory regions.

Publications:

Seif, I., Khoury, G. and Dhar, R.: BKV splice sequences based on analysis of preferred donor and acceptor sites. Nucl. Acids Res., 6:3387-3398, 1979.

Seif, I., Khoury, G. and Dhar, R.: The genome of human papovavirus BKV. Cell 18:963-977, 1979.

Aloni, Y., Dhar, R. and Khoury, G.: Methylation of nuclear SV40 RNAs. J. Virol. 32:52-60, 1979.

Alwine, J., Dhar, R. and Khoury, G.: A small RNA induced late in SV40 infection can associate with early viral mRNAs. PNAS 77: 1379-1383, 1980.

Seif, I., Khoury, G. and Dhar, R.: A rapid enzymatic DNA sequencing technique. Determination of sequence alterations in early SV40 temperature sensitive and deletion mutants. Nucl. Acid Res., 1980, in press.

Dhar, R., McClements, W., Enquist, L. and Vande Woude, G.: Terminally repeated sequence (TRS) of integrated Malony Sarcoma Provirus: Nucleotide sequence of TRS and its host and viral junctions. Proc. Natl. Acad. Sci. USA, 1980, in press.

Khoury, G., Alwine, J., Dhar, R., Goldman, N., Gruss, P., Jay, G., Segal, S. and Seif, I.: The role of RNA splicing in regulation of viral gene expression: Mobilization and reassembly of genetic information. Miami Winter Symposium, Academic Press, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04913-03-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Differentiation of Murine Teratocarcinoma Cells Permits SV40 Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Shoshana Segal	Senior Staff Fellow	LMV NCI
OTHER:	G. Khoury	Acting Chief, LMV	LMV NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.35	1.1	.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Infection of differentiated mouse embryo cells by simian virus 40 (SV40) leads to the production of the early mRNAs and the tumor (T) antigens that they encode. In contrast, undifferentiated F-9 murine teratocarcinoma cells do not support these early stages of the SV40 cycle. This block results at least in part, from the inability to accumulate stable processed early SV40 mRNAs. It has recently been shown that vitamin A and its derivatives can induce in vitro differentiation of stem cells. Undifferentiated F-9 cells, upon treatment with a low concentration of retinoic acid, exhibited pronounced morphologic changes as well as the appearance of the H-2 surface antigens. After differentiation, the susceptibility of F-9 cells to SV40 infection could be demonstrated by the appearance of large T- and small t-antigens, as shown by immunofluorescence and immunoprecipitation. Furthermore, S1 nuclease mapping of early SV40 transcripts confirmed the presence of the two spliced early mRNAs. These results indicate that the undifferentiated F-9 stem cells contain the genetic information needed for generating stable processed early SV40 mRNAs but are blocked in the production of functional species.

PROJECT DESCRIPTION

Objectives:

The study and analysis of the relationship between cell differentiation and gene expression as monitored by papovavirus infection.

Major Findings:

Studies on the absence of SV40 gene expression in undifferentiated murine F-9 teratocarcinoma cells revealed that these cells can transcribe SV40 DNA into full length, polyadenylated, precursor RNA molecules which represent nonspliced early transcripts. This finding suggests that at least one block to SV40 gene expression in F-9 cells is related to the inability to process the primary transcipts. In contrast, if F-9 cells are treated with retinoic acid which induces their differentiation in vitro and then are infected with SV40 or polyoma virus, the cells support the growth of polyoma virus as well as the abortive infection by SV40. We identified by immunofluorescence and immunoprecipitation large and small SV40 T-antigens produced in the infected differentiated cells.

Proposed Course:

The expression of the cellular genes (such as rat and human insulin) in undifferentiated and differentiated F-9 cells will be examined. Our purpose is to determine whether the inability to splice SV40 primary transcripts in undifferentiated cells is virus-specific or a general phenomenon linked to cellular differentiation. We will also try to determine if adenovirus genes can be expressed in undifferentiated F-9 cells and whether Ad2 RNA is processed.

Publications:

Segal, S., Levine, A.J. and Khoury, G.: Evidence for nonspliced SV40 mRNA in undifferentiated murine teratocarcinoma cells. Nature, 280:335-337, 1979.

Segal, S. and Khoury, G.: The regulation of SV40 Gene Expression in Non-permissive Cells. ICN-UCLA, 1980, in press.

Segal, S. and Khoury, G.: Differentiation as a requirement for simian virus 40 gene expression in F-9 embryonal carcinoma cells. Proc. Natl. Acad. Sci. 76:5611-5615, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04914-03-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Integration, Excision and Rearrangement of Genetic Information

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lynn Enquist	Research Scientist (PHS)	LMV NCI
OTHER:	K.D.-Thompson	Senior Staff Fellow	LMV NCI
	K. Umene	Fogarty Fellow	LMV NCI
	G. Vande Woude	Chief, VTBS	LMV NCI

COOPERATING UNITS (if any)

R. Weisberg, LMG, NICHD, NIH
N. Sternberg, FCRC, Frederick, MD
A. Landy, Brown University
T. Platt, Yale University

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our principal effort is directed to understanding the mechanisms by which novel DNA rearrangements occur in vivo. We have concentrated on two major systems: bacterial viruses capable of integration and excision and herpes simplex virus (HSV). Phage lambda is used for both in vivo and in vitro recombination systems and for cloning DNA fragments of HSV. We are analysing the mechanism by which phage lambda integrates through genetic manipulation of the integrase enzyme as well as through isolation of phages with altered integration sites. We are using lambda-HSV recombinants to define and characterize regions of HSV DNA rearrangement. We are analyzing one HSV fragment that is very unstable when propagated in E. coli. Similarly, we are studying a cloned DNA fragment carrying an integrated MSV proviral genome (Moloney Sarcoma Virus - transformed mink cells) that is highly unstable in E. coli. Techniques to isolate, characterize and quantitate deletion formation in DNA fragments carried by phage lambda vectors have been established. A fine structure deletion map of the HSV Us region has been constructed using lambda-HSV hybrids.

PROJECT DESCRIPTION

Objectives:

To understand how a virus inserts its genome into the host genome; to understand how specific DNA sequences are recognized by insertion-excision enzymes; to study regulation of virus encoded recombination functions; to isolate sites on HSV DNA involved in genomic rearrangement. To utilize prokaryotic technology to analyze eukaryotic genomes.

Major Findings:

I. The mechanism of phage integration and excision: a) we have characterized a mutation (xin = xis independent) that enables the phage to exise in the absence of the normally required excisionase product and in the absence of the normally required host factors. Lambda xin mutants integrate more efficiently than lambda$^+$ into secondary attachment sites as well as promote phage deletions at a high rate. b) We have improved the blot-hybridiztion analysis of lambda integration into unselected secondary sites and have found several frequently used sites rather than one major secondary sites. These sites will be used in comparison to the primary site to determine the nucleotide sequence that defines a strong and weak attachment site. c) About 1% of secondary site insertions inactivate genes (i.e. are auxotrophs) and the most frequent insertion inactivates the proline operon. DNA sequence analysis of this site was hampered by the discovery of an apparent second proline insertion site 300 bp away from the first. The improved blot-hybridization analysis subsequently revealed both insertions were at the same site and that one integrated phage had an IS2 insertion within 300 base pairs of the left prophage attachment site. This information enabled us (with T. Platt, Yale University) to sequence the proline integration site. d) We had characterized two rare insertions within the galactose operon that behaved genetically as if they were at different sites, but by blot-hybridization were within 50bp of each other. Detailed genetic studies (with R. Weisberg, NICHD) plus DNA sequence analysis (with A. Landy, Brown University) demonstrates that both insertions occured using the same site, but that the point of genetic exchange within the site was different. Excision of the lambda prophage from one of these sites generates changes called site affinity mutations both in the gal site and in the phage attachment site. Mutant gal sites have a much higher affinity than the normal gal site for insertion of wild type lambda. Mutant phage reinsert with lower than wild type efficiency into the primary bacterial attachment site. DNA sequence analysis indicates the two prophages differ by a shift of 4 to 7 bases in the prophage host-junction points. The site affinity mutations are generated by excision of one prophage using the sequences utilized by the other for integration. These mutations give insight into the mechanism of attachment site evolution as well as delineating those DNA sequences responsible for attachment site function. e) We have discovered a new region involved in regulation of integrase expression. This region is immediately to the left of the phage attachment site. Normally the cII/cIII gene products are required for expression of lambda repressor and lambda integrase through activation of two

separate promoters. When the region to the left of the phage attachment site is removed, lambda integrase, but not lambda repressor, expression becomes independent of cII/cIII. The deleted region is downstream of the integrase promoter and structural gene and conveys cII/cIII independence only in cis and not in trans. Studies on the nature of integrase mRNA and protein made under these conditions are underway.

II. Herpes simplex virus rearrangements: we have cloned a variety of EcoRI fragments from defective interfering particles of HSV-1 (strain Patton). These fragments all contain permutations of the terminal region of the HSV S segment. The entire unique S (Us) segment of normal HSV-1 was cloned in lambda to facilitate analysis of the defective interfering DNA. This normal fragment was subsequently found to be unstable during growth in E. coli. The frequency of spontaneous deletions was from 100-1000 fold higher for this fragment than for another HSV fragment of comparable size. The cloned Us fragment has about 700 bp of inverted repeat sequence at its termini and 90% of the deletions analysed ($^{26}/_{29}$) have one endpoint at or near one of these repeats. The other endpoint has no preferred position. A region of about 2000 bp within the fragment shows size heterogeneity in several independent isolates during growth in E. coli. The changes seem to be due to small (100-200 bp) deletions. Coincidently, this region is also involved in defective HSV formation. The deletions once formed are stable and we have collected and mapped more than 50 independent deletions of the Us fragment. A variety of lambda genetic methods have been used to select and identify deletions. In collaboration with N. Sternberg (FCRC) and R. Weisberg (NICHD), we have developed a simple and rapid system to select deletions in cloned fragments of lambda Ekl vectors. This method is based on the requirement of the lambda capsid protein, pD, for lambda genomes of 41kb or larger and the ability of phages with genomes of 41kb or less to grow in the absence of pD. The use of D amber mutations in lambda vectors facilitates propagation and selection of deletions in suppressor plus and minus E. coli.

III. MSV site specific recombination. We have characterized the site specific deletion that occurs in E. coli during propagation of a lambda vector carrying an integrated copy of the MSV provirus. While this deletion occurs frequently (10-50% of total progeny) other deletions involving nearby MSV sequences are exceedingly rare. The site specific deletion occurs in recA hosts. The deletion occurs between two 588 bp direct repeats of MSV DNA that bracket integrated provirus. The resulting phage carries a single copy of the 588 bp repeat. DNA sequence analysis of the 588 bp region revealed an 11 bp inverted repeat at the termini of each 588 bp repeat. The general structure and behavior of this sequence in E. coli is like that of insertion sequences (IS).

Significance to Biomedical Research and the Program of the Institute:

The rearrangement of genetic information by integration of one segment of DNA into another is widespread in prokaryotes and eukaryotes. A specific example is the insertion and excision of viral DNA. Moreover, such events

are known to change the physiology of the host organisms. It is highly likely that the successful symbiotic relationship of host-virus in the microbial system we employ has an analogy in the relationship between certain animal viruses and the cells of higher organisms. Moreover, an understanding of the control mechanisms operative in these easily studied microorganisms has obvious relevance to an understanding of regulatory elements in higher organisms. Finally, the application of recombinant DNA technology and microbial systems to the analysis of the large DNA genome of herpes simplex virus should provide new insight to the biology of this medically important virus.

Proposed Course:

We will continue the genetic and biochemical analysis of protein-DNA interaction using lambda integrase variants and specific attachment site mutations. Secondary attachment sites and the new control region to the left of the phage attachment site will be studied using DNA sequence analysis (T. Platt, Yale University). The HSV S region will be analysed in detail in vivo using site-directed mutagenesis and cDNA cloning of mRNAs localized with the S region. The deletion library of the S region will be used to map mutations by marker rescue. DNA sequence studies on the HSV regions involved in genomic rearrangement will be initiated.

Publications:

Enquist, L.W. and Szybalski, W.: Coliphage lambda as a safe vector for recombinant DNA experiments. In: Viruses and Environment. Academic Press 1978, pp 625-652.

Vande Woude, G.F., Oskarsson, M., McClements, W.L., Enquist, L.W., Blair, D., Fischinger, P.J., Maizel, J.V. and Sullivan, M.: Characterization of integrat Moloney sarcoma proviruses and flanking host sequences cloned in bacteriophage lambda. Cold Spring Harbor Symposium, 1980, in press.

Vande Woude, G.F., Oskarsson, M., Enquist, L.W., Nomura, S., Sullivan, M. and Fischinger, P.J.: Cloning of integrated Moloney sarcoma proviral DNA sequences in bacteriophage lambda. Proc. Natl. Acad. Sci. USA, 76: 4464-4468, 1979.

Enquist, L.W., Vande Woude, G.F., Wagner, M., Smiley, J.R. and Summers, W.C.: Construction and characterization of a recombinant plasmid encoding the gene for the thymidine kinase of Herpes simplex type 1 virus. Gene 7:335-342, 1979.

Sternberg, N., Hamilton, D., Enquist, L. and Weisberg, R.: A simple technique for the isolation of deletion mutants of phage lambda. Gene 8:35-51, 1979.

Enquist, L.W.: Special derivatives of phage lambda and their use for cloning fragments of complex genomes. In: Gene Structure and Function, Ohio State University Press, in press.

Enquist, L.W. and Denniston-Thompson, K.: Editors, Recombinant DNA. In Umbreit, W.W., Series Editor: Benchmark Papers in Microbiology. Stroudsberg, PA, Dowden, Hutchinson and Ross, 1979, in press.

Brownell, G.H., Enquist, L.W. and Denniston-Thompson, K.: The molecular characterization of the nocardiophage ØEC genome. In Schaal, K. and Pulverver G. (Eds.): The Biology of Actinomycetes. Berlin, Springer-Verlag, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04929-12-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Herpesviruses: Immunologic and Biologic Studies in Relation to Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of the project is to study the relationship between herpesvirus and human cancer. The major topic of interest concerns the mechanism of in vitro cell transformation by herpes simplex virus (HSV) and the putative co-carcinogenic role of HSV reflected in its ability to alter expression of endogenous type C virus information.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives:

To study the mechanism of cell transformation by herpes simplex virus (HSV), including the ability of HSV to alter the expression of endogenous type C virus information. To prepare monoclonal antibodies against HSV virion and nonvirion proteins and to develop sensitive radioimmunoassay procedures for use in seroepidemiologic studies with human herpesvirus.

Methods Employed:

Standard tissue culture, virologic, and biochemical methods were employed with modification as required.

Major Findings:

1. Transformation by HSV. Initial studies to delineate the mechanism of transformation by HSV using a Balb/c-derived mouse cell line (10E2) were completed. It was concluded that transformation of 10E2 cells by HSV resulted in the cells acquiring phenotypic properties comparable to those expressed by cells transformed spontaneously, but significantly different from cells transformed by SV40 (Cancer Res., in press). Attempts to detect HSV-specified proteins or nucleic acid in transformed cells were consistently negative, although the possibility that the cells retained a relatively small segment of the viral genome was not excluded. The results with 10E2 cells were consistent with two possibilities: 1) transformation by HSV does not require retention and expression of viral DNA, or 2) a segment of the HSV genome is retained in transformed cells, but it does not function to alter the cells' phenotypic properties as occurs with cells transformed by SV40.

   In addition to morphological transformation, HSV can induce biochemical trasnformation whereby the viral thymidine kinase (TK) gene is introduced into TK^- cells. The question of whether biochemical and morphological transformation by HSV are related events has yet to be definitively answered, although one report suggests that a fragment of viral DNA containing the TK gene is unable to morphologically transform TK^+ cells. Studies were initiated to determine more precisely the relationship between HSV-induced biochemical and morphological transformation. Initial studies were directed towards determining whether biochemically transformed cells are also morphologically transformed and tumorigenic. These studies required a nontransformed TK^- cell which was not available, since the widely used LTK^- mouse cells are already transformed and tumorigenic in nude mice.

   The 10E2 cells were selected for mutagenesis with BUDR to obtain a nontransformed TK^- cell. The cells were exposed to increasing concentrations of BUDR and were subcultured as required. After approximately a 12-month period, the cells were resistant to BUDR concentrations up to 100 µg/ml. When tested for growth in selective HAT medium and uptake into DNA of ^3H-thymidine, however, the BUDR resistant cells showed low levels of

residual TK activity. To completely eliminate this TK activity, the BUDR resistant cells were made resistant to IUDR. This required an additional three months until the cells were resistant to concentrations up to 100 µg/ml of IUDR. The IUDR resistant cells were cloned, maintained on IUDR, and tested for spontaneous reversion to a TK^+ state. Up to their 10th subculture, the cells' frequency of spontaneous reversion was approximately 10^{-6}. Subsequent to their 10th subculture, the reversion frequency was $<10^{-7}$. Subclones maintained in the absence of IUDR have shown no reversion to a TK^+ state when 10^6 cells were tested for growth in HAT medium. Two of three initially isolated clones (B and B1) proved tumorigenic in nude mice, while the third clone (clone B2 has remained non-tumorigenic for over 20 subcultures.

IUDR-resistant cells were infected with uv-irradiated HSV (uv-HSV), grown in selective HAT medium and colonies isolated for further testing. The TK^+ status of these cells was confirmed by demonstrating their ability to grow in HAT medium and their ability to incorporate ^{3}H-thymidine. The distinction between cellular TK and viral-coded TK was determined either by isoelectric focusing and neutralization with anti-HSV TK serum or by a modification of the method reported by Summers et al. which takes advantage of the fact that the viral-coded enzyme can utilize iododeoxycytidine (IdC) more efficiently than can the cell enzyme. Based on these two tests, over 100 TK^+ colonies were classified as being either spontaneous revertants or HSV transformants. As expected, several HAT resistant colonies isolated from early passage IUDR-resistant cells proved to be spontaneous revertants, while all HAT resistant colonies isolated from high passage cells were HSV transformants.

When tested in nude mice, 0/7 spontaneous revertants and 6/39 HSV transformants isolated from tumorigenic clone B and B1 cells were tumorigenic, while 45/46 TK^- clonal isolates from clone B and B1 cells provied tumorigenic. Based on these findings we concluded that 1) biochemical transformation by HSV does not result in morphologically transformed tumorigenic cells, and 2) biochemical transformation by HSV does not select for pre-existing tumorigenic cells in a TK^- cell population.

Several tumors induced by TK^- cells, spontaneous TK^+ revertants or HSV transformants were grown in culture and the TK status of the tumor cells shown to be the same as that of the parent cells used for initiation of the tumors. Of interest was the observation that transformed TK^- cells or spontaneous TK^+ revertants as well as HSV biochemically transformed cells which were tumorigenic in nude mice failed to form tumors in conventional (immunocompetent) mice. This differs from previous results with transformed 10E2 cells (spontaneous and HSV induced) which were tumorigenic in both nude and conventional mice. One possible explanation for these results is that during mutagenesis to a TK^- state, the cells were also mutagenized at other sites which resulted in expression of new antigens which function to cause rejection in conventional animals. This is presently under investigation.

In addition to testing biochemically transformed cells, TK$^-$ 10E2 cells were tested for tumorigenicity following morphologic transformation with uv-HSV. These cells were maintained in the absence of HAT medium. Tumorigenically transformed foci were isolated and tested for TK activity. In all cases, the HSV-transformed cells were characterized as being TK$^-$ with no evidence of viral TK or other proteins. Consequently, TK$^-$ cells tumorigenically transformed by HSV do not show evidence of retaining the viral TK gene. We conclude that biochemical and morphological transformation by HSV are unrelated.

2. Truncated T-Antigen in SV40 Transformed Cells. 10E2 cells transformed by SV40 were tested in parallel with HSV-transformed cells to determine differences in the properties of cells transformed by two DNA viruses. As part of this study, T-antigen expression in the SV40 transformed cells was assessed by immunoprecipitation of ^{35}S-methionine labeled cells using hamster SV40 tumor-bearing sera. Both the large T- (~90,000 mw) and small t- (~17,000 mw) antigens were precipitated with this sera as was a ~55,000 mw non-virus coded protein. Approximately 10% of the transformed cell foci showed a protein band at approximately 70,000 mw in addition to the 90,000 and 17,000 mw proteins. The cells from one of these foci were cloned in agar, agarose, or by isolated well-dispersed colonies. Of 29 clones so isolated, 28 showed the same protein pattern as did the parent cells (17,000; 70,000; 90,000 mw proteins) but one clone (2-5c) showed the 17,000 and 70,000 mw proteins but not the 90,000 mw protein. This protein pattern was confirmed by testing the 2-5c cells several times over a period of approximately one year (50 subcultures). Although clone 2-5c was not tumorigenic in nude mice when initially tested, tumor formation was observed after the cells had been subcultured 32 times. These tumors were removed and explants grown in vitro. The protein pattern of the tumor cells was identical to that of the parent cells in expressing the 17,000 and 70,000 mw proteins but not the 90,000 mw protein.

The 70,000 mw protein in 2-5c cells is related to the large T-antigen based on the following findings: First, sera from hamsters bearing SA12 virus transformed cells was shown to react with SA12 and SV40 large T-antigens, but not with small t-antigens. This sera precipitated the 70,000 mw protein expressed in 2-5c cells. Second, peptide maps showed that the 70,000 mw protein was related to the 90,000 mw T-antigen in SV40 transformed cells, but was different from the 17,000 t-antigen. Finally, in collaboration with Dr. G. Khoury, the cytoplasmic RNA in 2-5c cells was shown to have a deletion in the area of SV40 RNA which codes for large T-antigen.

In order to determine whether 2-5c cells contained a rescuable viral genome, the cells were fused with Vero cells using PEG. In two attempts, virus was rescued from the 2-5c cells. The rescued virus when grown in BSC-1 cells produced the normal large T- (90,000 mw) antigen but not the truncated 70,000 mw protein. The rescued virus was also used to transform 10E2 cells and 10 foci were isolated. The cells in 9 foci showed the normal large T- and small t-antigen pattern, while the cells from one focus showed the 70,000 mw protein in addition to normal large and small

T-antigens. Consequently, the 2-5c cells must contain an intact viral genome, although the cells express only a truncated large T-antigen. Of several possibilities which could explain these findings, two seem most probable. First, the 2-5c cells may contain an intact viral genome which is not being expressed and a defective genome which is being expressed. The intact viral genome is rescued by fusion with permissive cells. Second, the 2-5c cells may contain only an intact viral genome, but due to some cell regulatory effect transcription of the early region of the viral genome is faulty and results in expression of a 70,000 mw rather than a 90,000 mw protein.

3. Characterization of HSV Nucleocapsid Proteins. Studies have continued to characterize the 40,000 mw (p40) protein associated with HSV nucleocapsids. Hybrids secreting monoclonal antibodies have been produced against p40s of HSV-1 and -2 (Zweig et al., J. Virol. 32:676, 1979). The anti-HSV-1 p40 monoclonal antibody shows type-specificity, while the anti-HSV-2 monoclonal antibody shows some cross-reactivity with HSV-1 p40. The monoclonal antibodies have been employed for developing a sensitive ELISA procedure for typing HSV isolates.

   Studies have been initiated to identify type-specific and cross-reactive peptides in p40. Following partial digestion with V8 protease and immunoprecipitation with human, guinea pig, rabbit, or monoclonal antibodies, precipitation of specific peptides was observed, and some of these peptides have tentatively been identified as containing type-specific or cross-reactive determinants. Interestingly, the human sera tested, thus far, show reactivity with several p40 peptides, indicating that humans make antibodies to more than one determinant on the p40 molecule.

4. Development of Hybridomas Producing Antibodies to HSV Proteins. Mice were immunized with HSV-1 and hybrids were prepared with mouse myeloma cells. Initial screening indicates that approximately 10% (80/800) of the hybrids seeded are producing HSV specific antibodies. These hybrids are presently being propagated for cloning and further characterization.

5. Type C Virus Activation by Fragments of Epstein-Barr Virus DNA. The activation of endogenous mouse type C virus by sonicated DNA from various human and non-human herpesviruses was reported previously (Boyd et al., Proc. Natl. Acad. Sci. USA 75:4558, 1978). In the case of HSV type 1, type C virus activation was shown to occur with specific cloned DNA fragments (Boyd et al., in press). Similar studies have been initiated using EcoR1 digested fragments of EB virus DNA (strain B95-8 and P3HR-1) in collaboration with Dr. M. Nonoyama. Of 11 fragments tested thus far, 3 proved positive for type C virus activation (fragments A,D,I). Additional studies are underway using cloned DNA fragments.

Significance to Biomedical Research and the Program of the Institute:

Elucidating the mechanism of cell transformation by HSV may reveal a situation whereby an infectious, normally cytolytic DNA virus affects expression of an endogenous, vertically-transformed RNA virus. If a co-carcinogen role for HSV proves valid, it would raise the possibility of transformation by a

hit-and-run mechanism whereby persistence in the transformed cells of HSV gene sequences would not be required.

The development of radioimmunoassay procedures should facilitate sero-epidemiologic studies relating human herpesvirus to various disease states.

Proposed Course:

Studies will continue to develop quantitative assays for measuring transformation by HSV. Activation of type C virus by cloned HSV and EBV DNA fragments will be pursued. The preparation of additional monoclonal antibodies to HSV specified proteins will continue and will be utilized for studying virus expression in transformed cells.

Publications:

Hampar, B.: Activation of the viral genome in vitro. In Epstein, M.A. and Achong, B.G., (Eds.): The Epstein-Barr Virus. New York, Springer-Verlag pp. 284-295, 1979.

Zweig, M., Heilman Jr., C.J., Rabin, H., Hopkins III, R.F., Neubauer, R.H. and Hampar, B.: Production of monoclonal antibodies against nucleocapsid proteins of herpes simplex virus types 1 and 2. J. Virol. 32:676-678, 1978.

Boyd, A.L., Enquist, L., Vande Woude, G.F. and Hampar, B.: Activation of mouse retrovirus by herpes simplex virus type 1 cloned DNA fragments. Virology (in press).

Hampar, B., Boyd, A.L., Derge, J.G., Zweig, M., Eader, L. and Showalter, S.D.: Comparison of properties of mouse cells transformed spontaneously, by uv-irradiated herpes simplex virus, or by SV40. Cancer Res. (in press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04999-03-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of the Control of Simian Virus 40 Transcription and Protein Synthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James C. Alwine	Staff Fellow	LMV NCI
OTHER:	G. Khoury	Acting Chief, LMV	LMV NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION ~~Division of Cancer Cause and Prevention~~
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of total cytoplasmic and polyadenylated cytoplasmic RNA from cells lytically infected with Simian virus 40 has demonstrated the presence of a small RNA, approximately 65 nucleotides long, which is induced late in lytic infection. This SV40-associated small RNA (SAS-RNA) is apparently specific in size and sequence and is not selected on columns of oligo (dT)-cellulose. It is homologous to a region of the early SV40 mRNAs (and to the late (L)-DNA strand) starting approximately 250 nucleotides from the 3' end of the early mRNAs (SV40 map position 0.21). The function of SAS-RNA in the viral cycle, as well as its source, are unknown at this point; however, its temporal expression, unique sequence and interesting region of homology within the SV40 genome suggest a possible role in the control of SV40 gene expression. In additional experiments, we have attempted to analyze those factors responsible for the relative expression of early and late SV40 mRNA. Among our findings, we noted: 1) the overproduction of early SV40 mRNA and concommitant fall in late transcription which accompanies the shift

of a tsA mutant to the nonpermissive temperature, is highly dependent on the monkey cell line. 2) The relative splicing of the primary early SV40 transcript to large-T mRNA and small-t mRNA is strongly influenced by the temperature of the cultures.

PROJECT DESCRIPTION

Objectives:

1. To examine the early transcripts from (a) permissive monkey cells lytically infected with temperature sensitive A gene mutants (tsA mutants) and wild type viruses; and (b) nonpermissive cells transformed by the same viruses. These transcripts are compared quantitatively and qualitatively (by splicing pattern) to determine differences in transcriptional patterns.

2. To examine the protein products of the early genes made in the same cells used in Objective 1. This will allow a correlation of differences in the expression of proteins with the transcriptional patterns.

3. To examine the patterns of early gene expression described in Objectives 1 and 2 using various permissive monkey cell lines and transformed cells from a variety of species. This will define differences in early gene expression which may be determined in part by host cell regulatory factors.

4. To examine the effects of the tsA mutation on late transcription in lytically infected cells.

Methods Employed:

Analysis of viral transcripts by various types of gel electrophoresis and electron microscopy. RNA-DNA hybridization techniques to determine amounts of various viral transcripts. Immunoprecipitation of ^{35}S-methionine labeled SV40 early proteins with subsequent elution and electrophoresis on SDS polyacrylamide gels.

Major Findings:

1. In all permissive cells tested during lytic infection by tsA mutants or WT SV40, there was an increased ratio of large T- to small t-antigen mRNA upon growing the cells at increased temperatures (e.g. 32° and 41°). Transformed cells show this same phenomenom. Both of these mRNAs are presumed to be derived by different splices from one precursor RNA. For this reason, we believe the data indicate control of gene expression at the level of processing as determined by the physiological state of the cells.

2. Late SV40 RNA synthesis appears to require the continuous presence of functional large T-antigen; however, a host factor(s) is also needed. Apparently, there is an interaction between large T-antigen and the host protein(s).

3. We have characterized a 70 nucleotide RNA which is complementary to a specific region of the SV40 genome. We believe this may be an important control element, and it is presently being studied.

Significance to Biomedical Research and the Program of the Institute:

The early proteins of SV40 are directly involved in the establishment and maintenance of the transformed state. Understanding of the mechanism of expression of these proteins at the most basic level is vital for elucidation of their function. The finding of a putative control element is important in this regard, for it could indicate a mechanism for synthesizing early proteins which are slightly altered in amino acid sequence and, thus, in function. Comparison of lytically infected and transformed cells will be of particular value in these studies. The indication in late RNA synthesis that a host cell factor is involved with large T-antigen is an important finding with regard to the mechanisms of viral gene expression.

Publications:

Alwine, J.C. and Khoury, G.: Effect of a tsA mutation on simian virus 40 late gene expression: Variations between host cell lines. J. Virol. 33, 920-925, 1980.

Alwine, J.C. and Khoury, G.: Control of SV40 gene expression at the levels of RNA synthesis and processing: Thermally induced changes in the ratio of the simian virus 40 early mRNAs and proteins. J. Virol., 1980, in press.

Alwine, J.C., Dhar, R. and Khoury, G.: A small RNA induced late in simian virus 40 infection can associate with early viral mRNAs. PNAS 77, 1379-1383, 1980.

Khoury, G., Alwine, J., Dhar, R., Goldman, N., Gruss, P., Jay, G., Segal, S. and Seif, I.: The role of RNA splicing in regulation of viral gene expression. Miami Winter Symposium, Academic Press, 1980 in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05100-02-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Analysis of Early SV40 and Ad2-SV40 hybrid virus transcription

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George Khoury	Acting Chief, LMV	LMV NCI
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	R. Dhar	Visiting Scientist	LMV NCI
	J. Alwine	Staff Fellow	LMV NCI
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COOPERATING UNITS (if any)

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SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.0	.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have examined hybrid viral RNAs synthesized in both human and monkey cells infected by three nondefective adenovirus-SV40 hybrid viruses. Most of the hybrid viral RNA molecules appear to initiate within adenoviral sequences, but are polyadenylated on their 3' end at the early SV40 mRNA polyadenylation site. The $Ad2^{+}ND_4$ stock of virus is not homogenous, but consists of two principal populations of viral DNA. Both populations contain a segment of SV40 DNA extending from SV40 map positions 0.63 to 0.11 in a left to right orientation at adenovirus map position 0.79. One population contains an intact SV40 segment while the other (representing 80 to 85% of the population) has a 500 base-pair deletion mapping from approximately 0.60 to 0.50 SV40 map units. This deletion encompasses the SV40 DNA segment which encodes the early SV40 splice sites. Cells infected by the mixed $Ad2^{+}ND_4$ population induce the synthesis of at least three major SV40-containing RNA molecules among the hybrid viral transcripts. The most abundant of these hybrid mRNAs appears only late in the lytic cycle, after the onset of viral DNA replication.

It contains an RNA splice junction which extends from a donor nucleotide within the adenoviral RNA sequences to an acceptor nucleotide in the early region of SV40. This SV40 acceptor site is remarkable in that its use has not been detected in the spliced viral mRNAs of SV40 infected or transformed cells.

PROJECT DESCRIPTION

Objectives:

To determine the factors responsible for the specific splicing of SV40 or hybrid viral transcripts.

Methods Employed:

Restriction enzyme analysis, DNA sequencing, Northern transfer technique, S1-nuclease analysis.

Major Findings:

A significant fraction of the $Ad2^{+}ND_4$ hybrid virus population contains a deletion which includes the sequences for the early SV40 splice sites. Among the hybrid mRNA molecules synthesized from $Ad2^{+}ND_4$ is one which contains a new hybrid virus splice junction extending from an adenoviral RNA (5') donor nucleotide to an SV40 (3') acceptor nucleotide. This splice junction (a) is the first documented hybrid splice site (b) containing an SV40 acceptor site not normally used and (c) is found only in molecules synthesized late after the onset of $Ad2^{+}ND_4$ DNA replication.

Significance to Biomedical Research and the Program of the Institute:

An understanding of the synthesis and stability of early SV40 mRNA is important, in general, to our knowledge of gene regulation and, specifically, to control of tumor (T) antigen production.

Proposed Course:

Most of these studies with Ad2-SV40 hybrid viruses have been completed. We will, in future studies, concentrate some effort on establishing an *in vitro* transcriptional system--hopefully with the potential to splice RNA molecules.

Publications:

Khoury, G., Gruss, P., Dhar, R. and Lai, C.-J.: Processing and expression of early SV40 mRNA: A role for RNA conformation in splicing. *Cell* 18:85-92, 1979.

Khoury, G., Alwine, J., Dhar, R., Goldman, N., Gruss, P., Jay, G., Segal, S., and Seif, I.: The role of RNA splicing in regulation of viral gene expression: Mobilization and reassembly of genetic information. *Miami Winter Symposium*, Academic Press, 1980, in press.

Khoury, G., Alwine, J., Goldman, N., Gruss, P. and Jay, G.: A new chimeric splice junction in adenovirus 2-SV40 hybrid viral mRNA. J. Virol. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05101-02-LMV

PERIOD COVERED
October 1, 1979 through September 31, 1980

TITLE OF PROJECT (80 characters or less)

Molecular Mechanisms for Malignant Transformation and Immune Surveillance of Foreign Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	1.0	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to understand the molecular mechanisms for the malignant transformation of cells and for the immune recognition of foreign and tumor cells. We have been studying the structure and function of tumor antigens involved in neoplastic transformation and in the organization and expression of genes coding for surface transplantation antigens.

PROJECT DESCRIPTION

Objectives:

1. Studies on the organization and expression of the genes coding for the histocompatibility antigens involved in allograft rejection.

2. Studies on the function of the transformation-specific protein p53 in murine sarcomas and leukemias.

Methods Employed:

Recombinant DNA techniques carried out under conditions specified by the NIH Guidelines were used in these studies. Nucleic acids were detected by combinations of hybridization, gel electrophoresis and electron microscopy. Proteins were analysed by immunological methods, in combination with polyacrylamide gel electrophoresis.

Major Findings:

1. We have chemically synthesized a short DNA fragment whose nucleotide sequence was derived from the amino acid sequence of a region within the murine H-2K^{D} histocompatibility antigen. This DNA fragment was used as a probe to identify by hybridization to a mouse library those genomic clones which carry a related nucleotide sequence. Clones have been isolated and DNAs from these clones have been tested for their ability either to select for H-2 mRNA or to inhibit the cell-free translation of H-2 mRNA. Positive clones have been identified and their nucleotide sequences are being determined. The availability of such clones will allow the study of the genetic complexity, polymorphism and expression of the H-2 genes.

2. We have identified a transformation-related antigen p53 in murine cells. The panel of transformed cells that was tested and found to be positive for the expression of p53 includes chemically-induced sarcomas, viral and radiation-induced leukemias, DNA and RNA virus-transformed fibroblasts, spontaneously-transformed fibroblasts, and teratocarcinomas. Normal cells do not express any detectable amount of this protein; they include primary embryo cells, primary adult lung fibroblasts, 3T3 cells, macrophages, spleen cells, and bone marrow cells. However, thymic cells were found to synthesize p53 and spleen cells can be inducd to express this protein by treatment with phytohemagglutinin. We are presently attempting to clone the gene for the p53 antigen for delivery to normal cells using SV40 as a vector in order to study its possible role in cell transformation.

Significance to Biomedical Research and the Program of the Institute:

1. The H-2K and H-2D loci of the mouse histocompatibility complex code for structurally-related membrane glycoproteins which determine compatibility in tissue or organ transplantation. The complexity in allograft rejection is the result of their genetic polymorphism; that is, the existence of multiple alleles at each individual H-2 locus. A conservative estimate

indicates that there are at least 56 alleles at the H-2K locus and 45 alleles at the H-2D locus, thus allowing for some 2,500 different combinations between these two loci alone. Since there is a high degree of heterozygosity at these loci (that is, the presence of different alleles at the two genes in the homologous chromosomes), most of these combinations probably do exist in the mouse population. In addition, we have come to recognize that these H-2 antigens also play an important role in the immune response to foreign antigens. Specifically, the H-2K and H-2D loci are known to restrict effector lymphocyte specificity in such a way that maturing lymphocytes learn to recognize foreign antigens associated with the H-2K and H-2D molecules of the sensitizing cell. Success in cloning these genes will allow us to study the genetic complexit polymorphism, and gene expression of the H-2 histocompatibility antigens.

2. The biochemical events responsible for the transformation of normal cells to malignancy have not been defined. A central question in this regard is whether different classes of etiological agents transform cells by uniquely distinct pathways. Certain chemicals, radiations and oncogenic viruses are known to induce neoplasms in laboratory animals. While each of these agents may act distinctly to initiate the transformation of cells, it is possible that once a cell is committed, by whatever mechanism, it will follow a common pathway leading to the loss of control of proliferation. Our identification of a transformation-specific protein common to malignancies of different etiologies allows us to test this hypothesis and provides us with a biochemical handle to study the molecular basis for transformation.

Publications:

Jay, G., Ferrini, U., Robinson, E.A., Khoury, G. and Appella, E.: Proc. Natl. Acad. Sci. USA 76:6562-6566, 1979.

DeLeo, A.B., Jay, G., Appella, E., Dubois, G.C., Law, L.W. and Old, L.J: Proc. Natl. Acad. Sci. USA 76:2420-2424, 1979.

Jay, G., DeLeo, A.B., Appella, E., Dubois, G.C., Law, L.W., Khoury, G. and Old, L.J: Cold Spring Harbor Symp. Quant. Biol. 44, 1979 (in press).

DeLeo, A., Jay, G., Appella, E., Dubois, G.C., Law, L.W. and Old, L.J.: Transplantation Proc., 1980 (in press).

Jay, E., Seth, A.K. and Jay G.: J. Biol. Chem., 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05102-02-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Generation of HSV-1 defective DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Katherine D.-Thompson	Senior Staff Fellow	LMV NCI
OTHER:	L.W. Enquist	Research Scientist (PHS)	LMV NCI
	G.F. Vande Woude	Chief, VTBS	LMV NCI

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Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
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INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

Detailed physical maps have been constructed of hybrid lambda bacteriophage carrying EcoRI fragments cloned from DNA of defective interfering particles (dDNA) of Herpes simplex virus type 1 (HSV1) (strain Patton). Among our 34 cloned isolates, two size classes were identified. The major class (8-9.5 kilo base pairs (kbp)) made up about 80% of the fragments of the dDNA population, while the minor class (6.5-8 kbp) made up the remainder. Members of the two general size classes could be separated into three structural groups. All groups contained restriction sites characteristic of the S region terminal repeat (TRs). Major class Group I contained dDNA EcoRI fragments with restriction sites characteristic of the right end of the unique S region (Us) in the prototype orientation. This group is typical of HSV dDNA characterized previously. The minor class representatives of Group I contained an apparent 2kb site-specific deletion in the TRs region. Group II contained dDNA EcoRI fragments with restriction sites characteristic of the left end of the Us

region in the prototype orientation. Group III contained sequences originating from noncontiguous sequences of HSV-1 DNA fused to the TRs. The Group II and III dDNA EcoR1 fragments represent novel arrangements of HSV-1 dDNA. Group III dDNA structure suggests that TRs can recombine with high frequency to other regions of the HSV-1 genome.

PROJECT DESCRIPTION

Objectives:

To determine the origin of HSV-1 dDNA fragments in the wild type HSV-1 genome and the structure of specific dDNA cloned fragments. These analyses can reveal how defective molecules of HSV are generated. The cloned dDNA fragments are used also to investigate the replication of herpes simplex virus.

Methods Employed:

Four methods are being used to study the fine structure of the HSV dDNA clones. The first is restriction endonuclease mapping of dDNA clones and of cloned DNA from the same region of the wild type HSV genome. These large dDNA EcoR1 fragments will then be subcloned, using other restriction enzyme sites, from the lambda phage vector into the plasmid vector pBR322. These small subclones will enable determineation of maps for restriction emzymes having 10-30 cut sites. Such a detailed restriction map will allow the determination of the nucleotide sequence of the interesting regions of the dDNA molecule. Both the Maxam and Gilbert chemical degradations as well as the newer nick translation and chain termination enzymatic sequencing techniques will be used. Southern transfer hybridization experiments is used to study the origin of HSV-1 dDNA.

Major Findings:

Previous examination of the structure of HSV-1 dDNA have utilized entire uncloned, heterogeneous dDNA populations. We have used recombinant DNA technology to study the structure of individual dDNA molecules within the heterogeneous population. This has enabled us to define three structural groups of Patton dDNA EcoR1 fragments distinguished by properties other than the size of the iterated sequence. The major class of EcoR1 fragments (8-9.5 kbp) represent about 80% of the mass of the dDNA population. Group 1 members of this class closely resemble dDNA molecules described for HSV-1 strain Justin. They are composed of tandem repeats of the right most 8-9.5 kbp of the HSV-1 genome. Thus, each iterated EcoR1 fragment contains the S region termina redundancy (TRs) and a variable amount of the right end of the unique S region (Us). The novel joint forming the tandem repeats of Patton Group I dDNA varies in location within Us over a region of 1.5 kpb.

The Group I minor class, Group II and Group III dDNA EcoR1 fragments derived from Patton dDNA have not been previously described. The first indication of their novel structure was revealed when the less abundant, minor class fragments (6.5-8 kbp) were shown to be composed of two distinctly different groups of EcoR1 fragments. One minor class is similar to the major class

Group I dDNA EcoRl fragments, but represents approximately 10% of the dDNA. Six out of seven isolates of this group contain an apparent site specific deletion within the TRs. Because the fragments were cloned directly from a population of HSV dDNA as well as purified by size prior to cloning, we can be confident that these deletions did not occur during growth in E. coli.

Seven major and minor class fragments were characterized that contained the TRs and unique DNA substituted for the Us sequences found in the above Group I class. These seven fragments fell into two groups.

In the Group II fragments (one from the major class and one from the minor class), the new DNA has characteristic restriction sites of the left end of Us. These EcoRl fragments appear to be derived from dDNA molecules that originate in one of two ways: i.e. from the terminus of an HSV 1 genome with Us in the inverted orientation, or from the L-S joint region with Us in the prototype orientation.

The Group III fragments are most unusual in that the unique DNA sequences do not originate from Us sequences contiguous with TRs. Instead, one member of this group contains L-region DNA sequences and others appear to contain segments from 17 kbp Us region that are not contiguous with the TRs.

A physical map of the genome of actinophage ØEC was constructed in order to develop this phage and its host, Rhodococcus erythropolis, as a cloning system for the higher prokaryotes. These higher bacteria include a number of pathogenic organisms (Nocardia asteroides and N. brasiliensis, Mycobacterium leprae and M. tuberculosis) which are closely related to the Rhodococcus. Since Rhodococcus has never been shown to be pathogenic in man or other animals, it is an ideal host for such a cloning system. We have constructed a restriction map of the genome of ØEC for the following enzymes: PvuII, XbaI, HpaI, BamHI, and XhoI. The genome size estimated from restriction maps (45 kbp) is in good agreement with measurements of the genome by electron microscopy (43 kbp). The ØEC genome is unusual in that it is resistant to cleavage by a number of enzymes that would be expected to cut it 10-11 times (e.g. Sal I, HindIII, EcoRl, Bgl II) whereas others cleave the genome more than 25 times (SacI, PvuI). The phage genome has cohesive ends, like coliphage lambda, suggesting that it is encapsidated by recognition of the termini rather than by a headful mechanism.

We have generated a number of deletion mutants of the ØEC genome to determine what regions are nonessential and can be used in cloning experiments. Some of these deletion mutants have removed restriction enzyme sites that will faciliate use of ØEC as a cloning vehicle.

Significance to Biomedical Research and the Program of the Institute

The portion of the genome which gives rise to dDNA has been shown to activate the expression of endogeneous C-type virus genome in mouse cells. This region also accelerates malignant transformation of mouse cells. The ability to clone antigenic determinants unique to the pathogenic Actinomycetes could implement the early detection and subsequent treatment of diseases such as

leprosy. A closely related host-vector cloning system should facilitate expression of cloned genes possessing antigenic moieties of these pathogens.

Proposed Course:

We intend to sequence a number of the novel joints from the three groups of HSV-1 dDNA molecules and to investigate the role of the terminal (approximately 280 bp) "a" sequence in the generation of dDNA. We will also sequence the terminus of the HSV-1 genome as well as the joint between the L and S segments of the genome. It has been determined that the "a" sequence is present in 8 of the cloned dDNA EcoR1 fragments. This characterization will be extended to include the remaining 26 dDNA EcoR1 fragments.

We will also sequence regions of Us that are involved in the generation of HSV-1 dDNA.

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de Wet, J.R., Daniels, D.L., Schroeder, J.L., Williams, B.G., Denniston-Thompson, K., Moore D.D. and Blattner, F.R.: Restriction maps for 21 charon vector phages. J. Virol. 33:401, 1980.

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Brownell, G.H., Enquist, L.W. and Denniston-Thompson, K.: The molecular characterization of nocardiophage ØEC. In Schaal K. and Pulverver, G., (Eds.): The Biology of the Actinomycetes. Berlin, Spring-Verlag, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05103-02-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure and Function of MSV Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William McClements	Staff Fellow	LMV NCI
OTHER:	G.F. Vande Woude	Chief, VTBS	LMV NCI
	D.G. Blair	Expert	LVC NCI
	P.J. Fischinger	Chief, TVS	LVC NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure and biological properties of two Moloney sarcoma provirus isolates have been characterized. The integrated form of proviruses, cloned from transformed cell genomic DNA, have been subcloned and molecularly manipulated to determine portions of the viral genome essential for transformation. Both proviruses have terminal repeated sequences (TRS) ≈600 bp in length, which contain putative RNA transcription control signals. The general structures of the TRS and the integrated provirus are similar to those of some bacterial transposable elements. The transforming activity of MSV has been located within the provirus by direct transfection of cells with cloned subgenomic DNA fragments of MSV. In addition to src , two leukemia derived sequences are required for efficient transformation: 1) approximately 150 bases immediately adjacent to the 5' side of src and; 2) one copy of the TRS, either 5' or 3' to src.

PROJECT DESCRIPTION

Objectives:

This project is to identify and characterize the proviral DNA sequences of Moloney sarcoma virus (MSV) responsible for efficient transformation. These sequences are isolated from cloned proviral MSV DNA and from the normal mouse chromosome.

Methods Employed:

DNA from normal or transformed genomes is digested with restriction endonucleases and analyzed by the Southern blotting technique. Recombinant DNA technology is employed to clone retrovirus proviral DNA, subgenomic fragments of provirus, and normal cellular DNA. Biological activity of cloned fragments was determined in a direct DNA transfection assay.

Major Findings:

The integrated proviral genomes of two Moloney Sarcoma Virus (MSV) isolates have been cloned in bacteriophage lambda. The integrated proviruses are colinear with their viral RNA genomes and have terminally repeated sequences (TRS). More than 25 subgenomic clones of the MSV proviruses have been cloned and tested for biological activity in a direct DNA transfection assay and have shown which MSV sequences are necessary for cellular transformation. Cloned intact ml and HTl MSV proviruses transform with an efficiency of 40,000-50,000 focus forming units per picomole of proviral DNA, and the majority of these transformed cells contain a rescuable viral genome. A cloned 2.1 kb internal fragment of MSV containing 1.2 kb of sarcoma virus specific sequences (*src*) and approximately 900 base pairs of leukemia virus derived sequences adjacent to the 5' end of src, transforms approximately 10,000-fold less efficiently than the intact genome. When leukemia virus derived sequences containing a single copy of the 600 base pair TRS are present at either the 5' or 3' end of this *src*-containing fragment, the transforming activity is stimulated 1000-fold. Cotransfecting cells with a mixture of cloned fragments, one containing the internal 2.1 kb *src* fragment and the other containing a single copy of the TRS, results in a 300-fold increase in transformation efficiency. Thus, in addition to *src*, MSV sequences in TRS, derived from the parental leukemia virus are required for efficient transformation. These leukemia derived sequences may serve as control elements for *src* expression. Nucleotide sequence analysis of the TRS reveals putative RNA transcription and polyadenylation signals (R. Dhar, #Z01-CP-04907-04-LMV). (TRS) which define the virus-host junctions. Nucleotide sequence analysis of both TRS elements (sequence analysis performed by R. Dhar, #Z01-CP-04907-04-LMV) and the bracketing host sequence of one MSV isolate shows that the first and last 11 base pairs (bp) of the TRS form a perfect inverted repeat and that the same four bp host sequence occurs at both virus-host junctions. Recombinant lambda-MSV phages grown in *E. coli* undergo a high frequency deletion of most of the MSV sequences which appears to be mediated by the TRS (Z01-CP-04914-02-LMV). The resultant deletion has been used to characterize a cloned normal host cellular sequence which bears a site for MSV integration.

MSV provirus integration occurs without major viral or host DNA rearrangement. Evidence that an additional MSV region is required for efficient transformation by src is suggested from: 1) biological studies with the cloned normal mouse cell sarc fragment (Z01-CP-05103-01-LMV); 2) by comparing differences in the conserved leukemia virus portions of three leukemia virus isolates and; 3) by specifically deleting sequences 5' to src in a plasmid subclones without diminishing transforming activity. By these analyses, all but approximately 100 to 150 leukemia virus nucleotides preceeding src can be considered nonessential for transformation. The requirement for the 100-150 5' leukemia virus nucleotides is inferred from the activation of efficient transforming activity by sarc when these sequence and a single TRS is added to its 5' end (Z01-C-05103-01-LMV). These few nucleotides are derived from the 5' envelope gene of the leukemia virus and could provide a splice acceptor site and/or a signal peptide function to sarc.

Significance to Biomedical Research and the Program of the Institute:

These studies include the first identification and characterization of a leukemia cellular DNA sequence involved in virus sequence required for efficient transformation by a cellular gene transduced into a retrovirus. This work also includes the first demonstration of a biological function for a retrovirus terminally repeated sequence; (i.e. stimulation of transformation) and the first complete nucleotide sequence analysis of a TRS and the host-virus junctions. The TRS may have RNA transcription elements which would affect the expression of src. Furthermore, the general structures of the TRS and the provirus itself are similar to bacterial transposable elements. Therefore models for transposition in bacteria may be useful in understanding proviral integration and the transposition of sequences in eukaryotes.

Publications:

Vande Woude, G.F., Oskarsson, M.K., McClements, W.L., Enquist, L.W., Blair, D.G., Fischinger, P.J., Maizel J.V., Sullivan, M.: Characterization of Integrated Moloney Sarcoma Proviruses and Flanking Sequences Cloned in Bacteriophage Lambda. Cold Spring Harbor Symp. of Quant. Biology (in press).

Oskarsson, M.K., McClements, W.L., Blair, D.G., and Vande Woude, G.F.: Properties of a Normal Mouse Cell DNA Sequence (sarc) Homologous to the src Sequence of Moloney Sarcoma Virus. Science, 207, 1222-1224, 1980.

Blair, D.G., McClements, W.L., Oskarsson, M.K., Fischinger, P.J., and Vande Woude, G.F.: The Biological Activity of Cloned Moloney Sarcoma Virus-Terminally Redundant Sequences May Enhance Transformation Efficiency. Proc. Natl. Acad. Sci. USA, 1980 (in press).

Dhar, R., McClements, W.L., Enquist, L.W., and Vande Woude, G.F.: Terminally Repeated Sequence (TRS) of Integrated Moloney Sarcoma Provirus: Nucleotide Sequence of TRS and its Host and Viral Junctions. Proc. Natl. Acad. Sci. USA, 1980 (in press).

McClements, W.L., Enquist, L.W., Oskarsson, M.K., Sullivan, M., and Vande Woude, G.F.: A Novel Deletion of Coliphage lambda Murine Sarcoma Virus Recombinants: Characterization of a Retrovirus Host Integration Site. J. Virol., 1980 (in press).

McClements, W.L., Blair, D.G., Oskarsson, M.K. and Vande Woude, G.F.: Two Regions of the Moloney Leukemia Virus Genome are Required for Efficient Transformation by src/sarc. In Fields, B., Jaenisch, R. and Fox, C.F. (Eds.): Animal Virus Genetics, ICN-UCLA Symposia on Molecular and Cellular Biology XVIII. New York, Acad. Press, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05104-02-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of Splicing in Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter Gruss	Expert	LMV NCI
OTHER:	G. Khoury	Acting Chief, LMV	LMV NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

We have been interested in the role of RNA splicing in the biogenesis of mRNA. As a model system a small DNA tumor virus (SV40) was utilized. This viral system, or previously constructed mutants thereof, has been used as a vector to insert heterologous DNA segments in order to identify nucleotide signals involved in the regulation of eukaryotic transcription. Of particular interest was the rescue of a splicing defective SV40 deletion mutant from which the late intron has been precisely removed. This mutant, which produces no stable late viral RNA was used as a recipient for the insertion of an isolated mouse β^{maj} globin intron 1 and its flanking sequences. The β^{maj} intron without 5' and 3' ends of the β^{maj} globin gene was inserted in both the sense and antisense orientation relative to the late SV40 transcription unit, at a site which differs from the location of the original 16S RNA intron. Cloned recombinants harboring the inserted intron in the sense direction give rise to stable mRNA. Thus, while the polarity of the intron is critical to its function, the species of origin and the relative position

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in the mRNA are not. These observations suggest that introns represent functional elements in the generation of stable mRNAs.

To further investigate the biochemical signals important in gene regulation, we have made recombinant molecules between SV40 and rat preproinsulin genes. The complete rat preproinsulin gene 1 has been cloned into a simian virus 40 (SV40) vector. Most of the late region of the viral vector, including the SV40 intervening sequences (introns) and all of the major splice junctions were deleted and replaced by the entire rat insulin gene. The recombinant molecules plus a temperature sensitive (tsA) helper virus were inoculated into monkey kidney cultures. The formation of stable transcripts of the insulin insert is as efficient as the production of late SV40 mRNA. Analysis of these transcripts indicated that the rat preproinsulin gene nucleotide signals involved in RNA splicing and poly(A) addition are used. The generation of a class of mRNA similar in size to authentic rat insulinoma mRNA suggests that the initiation signal (promotor), of the insulin insert is also functional. Significant quantities of a protein immunoreactive with anti-insulin antibodies were detected. SDS-polyacrylamide gel electrophoresis in conjunction with competitive binding by bovine proinsulin suggested that this protein was rat proinsulin. The fact that the bulk of the protein was observed in the medium indicates possible secretion of the proinsulin.

PROJECT DESCRIPTION

Objectives:

The project aims to study gene expression in mammalian cells using SV40 as a vector for the insertion of cloned eukaryotic genes or portions thereof in order to elucidate transcriptional or posttranscriptional mechanisms for the expression of the inserted genes. The main objective is construction of recombinant molecules with subsequent deletion of essential regions to determine nucleotide sequences involved in initiation and splicing of the transcripts.

Methods Employed:

1. Construction of recombinant molecules: restriction endonuclease excision of DNA fragments; modification of the ends by addition of oligonucleotide "linkers"; ligation of required DNA fragments; cloning of altered genomes by complementation with helper virus.

2. Mapping of the recombinant genomes. Analysis was performed with restriction endonucleases.

3. RNA-analysis. In order to determine the stable transcripts the nuclease S-1 technique and a modification with Exo7 nuclease was used. In addition, "Northern" blots were hybridized with several nick-translated probes. Also, reverse transcription was performed in order to determine the 5' end region of mRNAs.

4. Immunoprecipitations of proteins and electrophoresis in SDS-urea polyacrylamide gels was done to identify the proteins synthesized by the SV40-rat preproinsulin recombinant.

5. Cloning of DNA fragments in pBR322 and single-strand bacteriophage fd.

Major Findings:

In the first study, we have examined directly the role of an isolated globin gene intron and its flanking nucleotides inserted into a previously constructed SV40 mutant missing precisely the intervening sequence of 16S mRNA (intron-minus mutant dl-2350).

As an intron source we selected a restriction enzyme fragment from the mouse β^{maj} globin gene, representing the entire intron 1 plus some flanking sequences. However, the 5' and 3' terminal sequences of the globin gene are not retained. Construction of the mutant involves insertion of the globin gene intron into the late region (0.72 map unit) of dl-2350. The site of insertion of 221 nucleotides upstream from the original splice junction for 16S mRNA. Thus, the location of the inserted intron differs from the original position of the intron for 16S RNA. While the experimental protocol for the insertion allowed ligation in both possible orientations relative to the late transcription unit, only the presence of an intron in the sense direction rescued stable mRNA. A correctly oriented intron can be removed in a splicing process, as indicated by the size of the resultant chimeric RNA. It should be emphasized, however, that some mouse flanking sequences for the globin intron 1 are preserved in these recombinant molecules and may be required for efficient splicing. The comparison of SV40 late introns with the βglobin intron 1 did not show extensive homology, with the exception of consensus sequences at the splice junctions. Nevertheless, the insertion of the globin intron resulted in the reconstitution of stable RNA . This experimental result provides strong support for the idea that the splicing event is coupled to the stability of the RNA. Accordingly, defined intron sequence appear less likely to be involved in a positive transcriptional regulation event.

In a second set of experiments, we have set the entire gene for rat preproinsulin 1 (rI_1) into the late region of SV40. The segment of SV40 deleted to accomodate the insertion of rI_1 included the known SV40 late splice sites, extending approximately from 0.72 to 0.14 map units, in a clockwise direction. Thus, splicing of RNA was presumed to be dependent on the intact rI_1 splice junction. Analysis of the RNA suggests that in fact, the rI_1 splice junction was active and presumably led to the generation of stable RNA molecules. It also appeared from this RNA analysis that the rat insulin polyadenylation signal, which was situated 800 to 900 nucleotides proximal to the late SV40 polyadenylation signal, was used preferentially. One class of transcripts appears to have a 5' end similar to authentic mRNA derived from rat insulinoma cells.

By immunoprecipitation, we were able to show that infection of AGMK cells by the recombinant SV40-rI$_1$ led to the production of rat proinsulin. The indentification of this polypeptide was based on comigration of the ^{35}S-labelled immunoprecipitated protein with a bovine proinsulin marker of similar size. The insulin immunoreactivity was also confirmed by radioimmune assay. These findings suggest that the rat preproinsulin hydrophobic leader peptide can be cleaved off in AGMK cells, but that these cells do not have the enzymatic machinery to remove the internal C-peptide. The presence in the tissue culture medium of significant amounts of protein immunoreactive with insulin antiserum can be attributed to the secretion of insulin-reactive polypeptides.

Proposed Course:

This project will continue with an emphasis on the genetic signals responsible for expression and modification of eukaryotic mRNAs.

Publications:

Gruss, P., Lai, C.-J., Dhar, R. and Khoury, G.: Splicing as a requirement for biogenesis of functional SV40 mRNA. Proc. Natl. Acad. Sci. USA 76, 4317-4321, 1979.

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Gruss, P. and Khoury, G.: The expression of rat preproinsulin genes in SV40 recombinant molecules. J. Supramol. Struc. Supplement 4, 485, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05151-01-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

SV40 DNA replication in vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Chikako Sumida-Yasumoto	Expert	LMV NCI
OTHER:	G. Khoury	Acting Chief, LMV	LMV NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A soluble cell-free system which catalyzes semi-conservative SV40 DNA replication of exogenously added SV40 form I DNA has been developed from African green monkey kidney or CV-1 cells infected with this virus. The DNA synthesis required Mg^{++}, ATP, all four dNTP, and exogenous SV40 form I DNA as a template and yielded SV40 form I and form II DNA as products. The reaction was inhibited by N-ethylmaleimide and salt, but not by ddTTP. These results suggest involvement of DNA polymerase alpha in SV40 DNA synthesis in vitro (as in vivo). This SV40 DNA-dependent SV40 replication also seems to depend on the product of A gene of SV40, i.e., SV40 large T-antigen. These findings provide for an in vitro complementation assay for SV40 large T-antigen.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives:

To understand the biochemical mechanisms of eukaryotic DNA replication by studying in vitro SV40 DNA replication as a model system.

Methods Employed:

Sucrose gradient centrifugation; isopycnic centrifugation; in vitro DNA replication assay; in vitro complementation assay for SV40 large T-antigen.

Major Findings:

A soluble extract from African green monkey kidney or CV-1 cells infected with SV40 carried out semi-conservative SV40 DNA replication of exogenously added SV40 form I DNA. Maximal DNA synthesis required Mg^{++}, ATP, all four dNTP, and exogenously added SV40 form I DNA as template. Sedimentation analysis of DNA products labeled in vitro showed the products to be SV40 form I and form II DNA. The reaction was resistant to ddTTP but sensitive to salt and N-ethylmaleimide. Reaction mixtures containing an extract prepared from cells infected with a tsA mutant of SV40 were thermolabile compared to the ones prepared from cells infected with wild type virus. Based on these observations, an in vitro complementation assay for SV40 large T-antigen has been devised which allows one to purify the SV40 large T-antigen based on its function in DNA replication.

Proposed Course:

More detailed study of in vitro SV40 DNA replication to understand the biochemical mechanisms of eukaryotic DNA replication.

Purification and characterization of the multi-functional protein, SV40 large T-antigen.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05152-01-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Analyses of the virus-virus interaction between different groups of oncogenic DNA viruses.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Neil D. Goldman	Staff Fellow	LMV NCI
OTHER:	G. Khoury	Acting Chief, LMV	LMV NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Experiments were carried out to determine the effect of adenovirus on SV40 replication, transcription and translation during coinfection of primary African green monkey kidney cells. Coinfection of these cells with SV40, concomitantly or prior to adenovirus infection, results in the abolishment of the block to adenovirus growth in monkey cells. Some evidence has pointed to the posttranscriptional block as a result of inefficient processing (splicing) of late adenovirus mRNA. The A gene product of SV40, T-antigen, has been shown to be responsible for this adenovirus "helper function." Although adenovirus can inhibit SV40 DNA replication, there is no effect on SV40 transcription and translation at a low adenovirus multiplicity of infection. Further studies indicated that SV40 could complement a conditional lethal DNA-negative adenovirus mutant for replication, and adenovirus could modulate the overproduction of early mRNA from an SV40 conditional lethal DNA-negative mutant at the restrictive temperature. It was also observed that at low stringency,

SV40 showed homology with adenovirus at the origins of replication. The results suggest that adenovirus can sequester T-antigen for its own utilization.

2. Further analyses of T-antigen are being carried out to study this protein's functions as they relate to structure.

PROJECT DESCRIPTION

Objectives:

To study the mutual utilization of tumor virus gene products by viruses from different groups.

Methods Employed:

DNA, RNA and protein extractions, polyacrylamide gel electrophoresis of DNA restriction enzyme fragments and transfer by the Southern technique, hybridization of RNA or DNA to DNA containing blots.

Major Findings:

Monkey cells are semi-permissive for adenovirus growth. Previous studies have indicated that the posttranscriptional block in monkey cells can be overcome by an SV40 gene product, the tumor (T) antigen. This effect of T-antigen may be at the level of processing (splicing) of adenovirus late mRNA and/or translation. Other findings have suggested that SV40 can complement adenovirus mutants temperature-sensitive for replication. We have studied the ability of adenovirus to regulate SV40 macromolecular synthesis, and ultimately the ability of adenovirus to functionally interact with SV40 T-antigen. During coinfection of monkey cells with adenovirus (MOI = 1-5) and SV40 (MOI = 10-20), the kinetics of SV40 DNA replication are dramatically inhibited coincident with the onset of adenovirus DNA synthesis. Although adenovirus is able to inhibit SV40 DNA synthesis we observed no effect of adenovirus on SV40 transcription or translation. These data suggest that adenovirus could sequester T-antigen possibly for its own use. Further experiments demonstrate that SV40 can complement Ad5 ts125 (conditional lethal DNA mutant) by enhancing the adenovirus mutant DNA synthesis by more than ten-fold at the nonpermissive temperature. In addition, adenovirus is able to modulate the overproduction of SV40 tsA58 early mRNA to a more normal (wild-type) level. The above data indicate functional interaction at the macromolecular level between DNA tumor viruses of different groups. This has prompted continued investigation on the properties of T-antigen, which may be reponsible for its functional activity.

Significance to Biomedical Research and the Program of the Institute:

These studies were initiated to provide additional insight into the mutual utilization of viral macromolecules by DNA tumor viruses from different groups. In particular, we are interested in the interaction of SV40 tumor (T) antigen, a protein required for transformation and tumor specific transplantation immunity, with DNA and other proteins.

Proposed Course:

This project will be continued with emphasis on the role of the structure of T-antigen and its relation to the many functions of this protein.

Publications:

Goldman, N. and Khoury, G.: Adenovirus regulation of simian virus 40 macromolecular synthesis. J. Virol. June, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05156-01-LMV

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Expression of MSV Genetic Information

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomas Gordon Wood	Staff Fellow	LMV NCI
OTHER:	G.F. Vande Woude	Chief, VTBS	LMV NCI
	K. Denniston-Thompson	Staff Fellow	LMV NCI
	R. Dhar	Staff Fellow	LMV NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Virology, Carcinogenesis Intramural Program

SECTION
Virus Tumor Biochemistry Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

RNA from both normal cells and cells transformed by Moloney sarcoma virus (MSV) have been analysed for the expression of viral specific sequences. The MSV genome contains sequences derived from Moloney leukemia virus (MLV) and a cellular host sequence (src). The latter sequence is responsible for transformation by MSV. Recombinant DNA bacteriophage lambda clones containing either the integrated provirus of two MSV isolates or the normal mouse cell sarc sequence from Balb/c mice have been used as probes in detecting the expression of genomic and subgenomic messenger RNAs in a variety of infected and uninfected cell lines. A direct comparison of the nucleotide sequence of the viral src and host sarc is in progress. This information in conjunction with the analysis of the representative messenger RNAs transcribed from these genes will be used in determining the genetic elements required for transformation.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objectives:

This project is to identify and characterize messenger RNA expression from MSV proviral DNA sequences. In particular, to identify any putative src or sarc specific messenger RNAs and to compare the genetic information encoded in these two genes with emphasis on determining those genetic elements required for cell-transformation.

Methods Employed:

Total cytoplasmic and polysomal RNA is isolated from normal and viral infected cells and separated into poly(A) containing and noncontaining fractions by affinity chromatography using oligo (dT) cellulose. RNA species are separated by electrophoresis on agarose - CH_3HgOH gels and analyzed by Northern blotting techniques. Recombinant DNA technology is employed to clone retrovirus proviral DNA, subgenomic fragments of provirus and normal cellular DNA sequences. These DNAs are then used to prepare ^{32}P-labeled nick translated probes for hybridization.

Sequence analysis of src and sarc is performed using recombinant DNA clones. The appropriate DNA is digested with restriction endonucleases and end-labeled using γ-^{32}P-ATP and the T_4 polynucleotide kinase exchange reaction. Labeled DNAs are sequenced using a combination of Maxim and Gilbert technique and the Sanger dideoxynucleotide techniques.

Major Findings:

Normal mouse DNA containing sarc cloned in bacteriophage lambda has been heteroduplexed with two isolates of MSV. Both heteroduplexes showed that the only homology existing between the normal cellular DNA and the two MSV isolates was located in the cellular sarc sequence and the viral src region. Digestion of the normal mouse cloned DNA with restriction endonucleases and analysis by Southern blotting technique using cDNA prepared from Moloney Murine Leukemia Virus (MuLV) as probe revealed a restriction fragment of approximately 4kb in size which was recognized by the MuLV cDNA probe. This sequence is located 2000 bases from the 3' end of the sarc gene. RNA from MuLV producing NIH3T3 cells does not contain an RNA sequence recognized by this DNA. However, 28S ribosomal RNA from rabbit reticulocytes was recognized in a Northern blot analysis using the cloned normal mouse DNA as probe. Mouse NIH3T3 cell 28S RNA is not similarily recognized in an identical analysis. R-loop analysis using 28S rabbit reticulocyte ribosomal RNA and the bacteriophage lambda cloned normal mouse DNA showed approximately 165 bases of shared homology between the two molecules. The source of the shared homology and its relationships to sarc is currently under investigation.

Significance to Biomedical Research and the Program of the Institute:

Understanding the mechanism by which a normal mouse gene (sarc) was acquired by Moloney leukemia virus and what activates the expression of this gene such that the resulting virus has the potential for cell transformation is of

paramount importance. Characterizing the messenger RNAs expressed by these viruses, determining the nature of the sequences surrounding the normal mouse sarc gene and comparing the nucleotide sequence of the viral src gene to the mouse sarc gene should provide us with some of the answers.

Publications:

Wood, T.C. and Arlinghaus, R.B.: Precursor polyproteins of Moloney murine leukemia virus. Biochem. Biophys. Acta. 565, 183-191, 1979.

Ng, V.L., Wood, T.G., Lyons, D.D. and Arlinghaus, R.B.: Characterization of intracellular precursor polyproteins of Moloney murine leukemia viruses. J. Virol., 1979, in press.

Wood. T.G., Lyons, D.D., Wg., V.L., Murphy, E.C. and Arlinghaus, R.B.: Characterization of viral polyproteins in cells transformed and producing Moloney murine sarcoma virus. Biochem. Biophys. Acta., 1980, in press.

Wood, T.G., Horn, J.P., Robey, W.G., Blair, D.G. and Arlinghaus, R.B.: Characterization of viral specified proteins present in NRK cells infected with a temperature-sensitive transformation mutant of Moloney murine sarcoma virus. Cold Spring Harbor Symp. on Quant. Biol., 1979, in press.

Horn, J.P., Wood, T.G., Blair, D.B. and Arlinghaus, R.B.: Partial characterization of a Moloney murine sarcoma virus 85,000 dalton polypeptide whose expression correlates with the transformed phenotype in cells infected with a temperature-sensitive mutant virus. Virology, 1980, in press.

SUMMARY REPORT

LABORATORY OF TUMOR VIRUS GENETICS

October 1, 1979 through September 30, 1980

The Laboratory of Tumor Virus Genetics has enjoyed the most productive year since its inception in 1975. The goal of the Laboratory at that time was to identify a viral gene(s) and gene product(s) involved in the induction of malignant transformation of fibroblasts and hematopoietic cells. Over the past five years, this goal has been realized. The transforming proteins of a mammalian sarcoma and a leukemia inducing virus have been identified, and the laboratory has now begun to investigate how these proteins effect oncogenic conversion. In addition to conducting studies to elucidate the biochemical pathways of malignant transformation, we have begun to define the biology and in vitro growth properties of pluripotent hematopoietic stem cells (CFU-S).

During the past year, we have extended our studies on the rat-derived Kirsten sarcoma virus (Ki-SV) and Harvey murine sarcoma virus (Ha-SV). In 1973, we discovered the fundamental mechanism by which strongly oncogenic retroviruses arose. Recombination between non-oncogenic retroviruses and cellular genes was found to generate RNA tumor viruses with new genes coding for transforming proteins. The transforming protein, p21, of Ki-SV and Ha-SV was identified last year. This year we have used molecular DNA clones of Harvey virus to definitively prove that p21 is the sole transforming protein of Ha-SV, and to locate its position on the Harvey sarcoma virus genetic map. More importantly, p21 was found to bind with high affinity to the guanine nucleotides, GDP or GTP. The p21 catalyzed metabolism of GTP is now being studied. p21 is strikingly different from the src protein, p60, of Rous sarcoma virus. The p60 utilizes ATP and phosphorylates tyrosine residues while the murine virus p21 will not use adenine nucleotides and phosphorylates theonine residues. Additionally, p21 is the first GTP-specific protein kinase to be described. As a GTP-specific kinase, the cancer-inducing p21 protein, is a biologically unique molecule. A normal homologue protein of p21 has been found in all normal vertebrate cells, including human cells. Qualitative and quantitative differences have been noted between the endogenous p21 and the viral src p21. We are studying the detailed biochemistry of each protein in an effort to define the molecular pathway involved in the p21 induction of cancer. The work uses the techniques of molecular cloning, sophisticated in vitro enzymology and cell biology. The comparative biochemistry of p21 of Harvey virus and p60 of Rous virus should provide biochemical assays to aid in the clinical differential diagnosis of various forms of human cancers. Both p60 and p21 are expressed in human cells, and it is reasonable to expect that some human cancers will be found to be caused by each protein.

The comparative biochemistry of these viral transforming proteins has provided us with two unique and distinct parameters with which to investigate human cancers. Phosphotyrosine containing proteins and GTP-specific protein kinase activity can now be explored as new tests for the differential diagnosis of human cancers. The research on p60 and p21 have given impetus to studies

to develop a rational biochemical basis for the classification of cancers. To date, it has been impossible to attempt such a biochemical classification. As the biochemistry of these and other transforming proteins evolves, a revolutionary change in the diagnosis of human cancers will occur.

For our studies on hematopoietic neoplasms, we have developed a system to study the proliferation of erythroid cells induced by three different leukemia-inducing viruses. Each virus induces a somewhat different form of erythroleukemia. The genes and gene products of each virus which induce erythroleukemia have been identified, and cell culture assays in mouse bone marrow cultures are being developed. Cellular markers for each form of erythroleukemia are being developed. We believe this will be a unique system for studying leukemogenesis and hematopoietic cell biology. Using molecular cloning methods, we can generate a variety of mutants in the viral genes that cause each form of erythroleukemia and thus dissect the steps that control the growth and differentiation of erythroid precursors. An even more exciting observation has emanated from studies on one of the erythroleukemia inducing viruses which apparently can cause the proliferation of normal hematopoietic stem cells in vitro. We have analyzed a cell line infected by this virus which was developed by T. M. Dexter. This permanent stem cell line, called 416B, seems to have been induced by one of our viruses. The cell line forms CFU-S colonies in mice, is not malignant, and can completely protect mice when they are reconstituted with it after otherwise lethal irradiation. We are intensively investigating what viral gene is responsible for this growth-promoting effect on the stem cell. The identification of this gene and its gene product is of obvious importance to biomedical research since the availability of such a gene product may allow the routine growth of pluripotent hematopoietic stem cells in unlimited quantities in cell culture. In collaboration with Dr. Dexter, we have also developed cell lines which are normal CFU-C granulocyte precursors. Thus, our research on hematopoietic neoplasms has taken us into studies on the mechanisms involved in both normal and abnormal stem cell proliferation. These studies have already produced both important intellectual and practical findings. The use of viruses in these studies is a fundamental necessity since we know of no other way to genetically manipulate hematopoietic precursor cells.

In the past year, the work of the Laboratory has gone from the area of pure basic research toward the area of applied research. The viral gene products involved in growth control of cells are being used (1) to develop tests for the differential diagnosis of human cancer, and (2) to develop techniques to grow unlimited quantities of hematopoietic stem cells for clinical use. In the coming years, we will attempt to increase the applicability of the major discoveries and successes of basic tumor virology to the diagnosis, treatment and prevention of human cancer.

During the past year, the Chief of the Laboratory was awarded the 1980 Eli Lilly Award for Microbiology in recognition of the sustained excellence of this intramural Laboratory over the past decade.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04846-08-LTVG

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Analysis of Viral Infection and Its Control at the Cellular Level

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. I. Gerwin	Chemist	LTVG, NCI
OTHER:	R. H. Bassin	Head, Viral Biochemistry Section	LTVG, NCI
	Y. Inoue	Visiting Fellow	LTVG, NCI

COOPERATING UNITS (if any)

D. Lowy	Medical Officer,	DCB, NCI
J. Levin	Chemist	LMG, NICHD
A. Rein	Frederick Cancer Research Center	

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Viral Biochemistry Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are utilizing a non-conditional polymerase mutant of B-tropic MuLV to study the mechanism of the reverse transcriptase reaction. This mutant retains some of the catalytic activity of the wild-type enzyme but does not synthesize DNA from the natural template. The nature of this block is being investigated. In addition, mutants of tropic MuLVs are being constructed using recombinant DNA technology. It is hoped that such mutants will enable us to correlate known viral genes and gene products with specific viral functions.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The goal of this project is an understanding at the molecular level of the life cycle of RNA tumor viruses, i.e., the expression of these viruses in cells infected both nonproductively and productively, as well as an understanding of the cellular control mechanisms applicable to these viruses.

The topics of present interest are:

(A) Analysis of the products of in vitro DNA synthesis by a mutant polymerase molecule. Further characterization of the mutant viral genome which codes for this protein.

(B) Generation and characterization of mutants of endogenous leukemia viruses.

Methods Employed:

(A) Enzymes are purified from disrupted virions and cells using affinity and ion-exchange chromatography and velocity gradients. Products of DNA synthesis by mutant and wild-type enzymes are compared by gel electrophoresis, DNA blotting techniques and filter hybridization. Kinetics of enzymatic reactions are analyzed.

(B) In order to create multiple mutants of an endogenous RNA tumor virus, it is necessary to recombine the DNA encoding the virus with that of an appropriate circular vector. After this clone is isolated, the DNA will be specifically cleaved by restriction endonucleases and full length linear molecules isolated. Short, defined sequences will be added to these molecules and the mutant DNA will be recircularized and used to infect bacteria. Bacteria will be screened and those containing mutants will be selected. These mutants will be studied for infectivity, for blockage at specific points in the RNA tumor virus life-cycle and for their ability to complement or be complemented by other defined RNA tumor virus mutants or genome fragments.

Major Findings:

(A) The altered polymerase molecule present in the mutant virions can copy synthetic templates but appears defective in its ability to copy the native viral genome.

(B) 1) The genome of an endogenous RNA tumor virus has been successfully inserted into a plasmid vector. The generation of mutants from this virus is in progress. 2) A defective leukemia virus has been isolated from a transplantable murine leukemia cell. This virus is defective in its structural

proteins and polymerase molecule but contains an envelope gene. The only immunologically reactive protein detected in cells containing this viral genome is the glycosylated precusor to the viral envelope glycoprotein.

Significance to Biomedical Research and the Program of the Institute:

(A) Further characterization of this polymerase mutant will increase our understanding of the mechanisms of RNA tumor virus replication. Identification of a sensitive portion of this reaction may reveal a phase of the viral life cycle which would provide a target for chemotherapy.

(B) There are few non-conditional mutants of mammalian RNA tumor viruses. The development of well characterized mutants will provide important tools for analysis of infection by RNA tumor viruses both *in vivo* and *in vitro*. The availability of such mutants as recombinant DNA clones will allow precise mapping of the defect on the genome and provide information as to the functional significance of known genes. Such information would be of importance in understanding how these viruses produce cancer.

Proposed Course:

(A) Comparison of reactions catalyzed by the mutant and wild-type polymerases will be performed. R-looping of mutant and wild-type genomes will be carried out in order to locate the defect on the viral genome.

(B) Mutants will be generated and analyzed both biologically and biochemically.

Publications:

Gerwin, B. I., Rein, A., Levin, J. G., Bassin, R. H., Benjers, B. M., Kashmiri, S. V. S., Hopkins, D., and O'Neill, B. J.: Mutant of B-tropic murine leukemia virus synthesizing an altered polymerase molecule. J. Virol. 31: 741-751, 1979.

Benjers, B. M., Bassin, R. H., Rein, A., Gerwin, B. I., and Duran-Troise, G.: Mechanism of restriction of murine leukemia viruses varies between different strains of Fv-1^n mice. Int. J. Cancer 24: 600-607, 1979.

Rein, A., Athan, E., Benjers, B. M., Bassin, R. H., and Gerwin, B. I.: Isolation of a replication-defective murine leukaemia virus from cultured AKR leukaemia cells. Nature 282: 753-754, 1979.

Bassin, R. H., Duran-Troise, G., Gerwin, B. I., Rein, A., and Barlow, B.: Studies on the mechanism of Fv-1 restriction in Balb/3T3 cells. In Ikawa, Y., and Odaka, T. (Eds.): Oncogenic Viruses and Host Cell Genes. New York, Academic Press, 1979, pp. 3-19.

Bassin, R. H., Gerwin, B. I., Levin, J. G., Duran-Troise, G., Benjers, B. M., and Rein, A.: Macromolecular requirements for abrogation of Fv-1 restriction by murine leukemia viruses. J. Virol. August, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04848-08-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

RNA Tumor Viruses: Replication, Transformation and Inhibition in Cell Cultures

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. H. Bassin	Head, Viral Biochemistry Section	LTVG, NCI
OTHER:	B. I. Gerwin	Chemist	LTVG, NCI
	A. Brown	Bio Lab Tech (Micro)	LTVG, NCI
	Y. Inoue	Visiting Fellow	LTVG, NCI

COOPERATING UNITS (if any)

J. Levin	LMG, NICHD	A. Panet	LEP, A
W. Turner	Howard University, School of Medicine, Dept. of Microbiology		
Y. Yang	Oak Ridge National Laboratory, Biology Division		

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Viral Biochemistry Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The molecular mechanism of Fv-1 restriction was investigated using a sensitive Southern gel transfer technique. In BALB/c cells, Form III linear DNA is synthesized in normal amounts by restricted viruses but conversion to Form I circular DNA is blocked. Restriction endonuclease studies on viral DNA synthesized in BALB/c cells indicate that the linear DNA produced under non-permissive conditions is defective, lacking two sets of 70 base pair repeated sequences. Fv-1^n cells differ in their ability to restrict B-tropic MuLVs. Thus, DBA/2 cells appear to block synthesis of Form I circular DNA by B-tropic MuLVs, while NIH cells restrict at an earlier step, blocking formation by linear DNA. A recombinant between N-tropic and B-tropic MuLV which is sensitive to Fv-1^b restriction but lacks the ability to abrogate has been partially characterized.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The primary objective of this effort during the past year has been an understanding of the molecular mechanism of Fv-1 restriction and of the abrogation phenomenon. Additional experiments on the effect of cell synchronization and inhibition of cell multiplication on viral DNA synthesis have been initiated.

Methods Employed:

Murine leukemia viruses are quantitated in Fv-1 restrictive and permissive cells by a variety of cell culture procedures including the S+L- focus assay, the XC test and, when necessary, assays for cell transformation. Additional assay techniques are developed as needed, as are stable mouse cell lines. Viral DNAs are analyzed by a highly sensitive modification of the Southern gel transfer technique coupled with restriction endonuclease mapping and transfection with viral DNA.

Major Findings:

Certain mouse cells are able to effectively restrict the replication of murine leukemia viruses by means of a highly specific mechanism mediated by the Fv-1 gene. The molecular basis of Fv-1 restriction has been investigated using permissive mouse cells, restrictive cells, and restrictive cells in which the Fv-1 gene product had been abrogated (see below). Although linear (Form III) proviral DNA is synthesized in normal quantitites in nonpermissive BALB/3T3 mouse cells, the formation of circular (Form I) DNA is blocked. The block in proviral DNA synthesis can apparently be overcome by infection of the nonpermissive cell with two restricted MuLV particles--the amount of circular DNA synthesized in nonpermissive cells varies with the square of the virus concentration. The data agree well with infectious center assays, where it has been shown that replication of MuLV in nonpermissive cells also requires infection with two virus particles.

Close examination of the linear form of viral DNA synthesized in nonpermissive cells using restriction endonuclease mapping techniques indicates that it is slightly smaller than the DNA synthesized in abrogated (permissive) cells. Two 70 base pair sequences are missing from the DNA synthesized in restrictive cells, one sequence from each of the terminally redundant regions. It seems likely then that Fv-1 restriction of a virus causes some modification or "error" in the complex process of transcription of viral information into linear DNA. This error results in the failure of viral DNA to circularize.

One of the more interesting features of the Fv-1 system concerns the ability of MuLVs to overcome restriction following infection with two or more

virus particles, one of which turns off or "abrogates" restriction. The mechanism of abrogation appears to involve an as yet unknown viral function, perhaps the early synthesis of a viral protein. Recent data indicate that (1) Fv-1^b restrictive cells chronically producing one kind of N-tropic MuLV (amphotropic) are still able to restrict a second kind of N-tropic MuLV (ecotropic) and (2) N-tropic MuLV is able to abrogate restriction for B-tropic virus in Fv-1^b x Fv-1^n hybrid cells but not in Fv-1^n cells. Together with our previous data on abrogation, these results indicate that abrogation may involve the action of a previously unknown viral protein which is synthesized only as a result of Fv-1 restriction.

Previous infectious center studies using Fv-1^n cells indicate that there are in reality a variety of restriction patterns, depending upon the cell line employed. A recent study comparing the quantity and quality of virus-specific DNAs synthesized by MuLVs in various Fv-1^n cell lines indicates that Fv-1^n DBA cells exhibit the same block information of circular DNA observed above in Fv-1^b BALB/3T3 cells. NIH cells, on the other hand, show an additional block in Form III (linear) DNA formation. These results, which agree well with earlier infectious center data, show that additional restriction mechanisms which modify viral DNA synthesis exist in mouse cells.

In an effort to isolate MuLV variants which differ in this response to Fv-1 restriction, recombinants between N-tropic and B-tropic MuLVs originally isolated by N. Hopkins, MIT, were examined. Of nine recombinants tested, one was of special interest because it was sensitive to Fv-1^b restriction but failed to abrogate. This recombinant should be especially useful in biochemical studies of Fv-1 restriction, especially since it can be used to infect nonpermissive cells at high moi's without a concomitant loss of restriction due to abrogation. The existence of a recombinant with these properties supports the idea that abrogation does not represent the synthesis of a known viral structural protein and may help to resolve some of the conflicting data which has been published regarding Fv-1 restriction.

The effect of various inhibitors of cell multiplication on viral DNA synthesis is also being studied using techniques similar to those described above for the Fv-1 restriction studies. MuLV infection of serum-deprived cells is blocked at the level of DNA synthesis, and addition of serum following infection results in a peak of both cellular and viral DNA synthesis some 17 hours later. Interferon also inhibits cell multiplication, but the effect is not absolute, and viral DNA appears to be synthesized. The effects of other inhibitors of cell multiplication are currently under study.

Significance to Biomedical Research and the Program of the Institute:

The mouse Fv-1 restriction system has at least two aspects which are of significance. In the first place, a study of the mechanism by which cells are able to restrict the formation of viral DNA will undoubtedly provide insight into the details of normal virus replication. Indeed the finding

that two 70 base pair regions are missing for the DNA synthesized in BALB cells by N-tropic MuLV has given some indication of how the DNA is synthesized. A second mechanism of restriction which in some way blocks the synthesis of linear viral DNA has also been observed in NIH cells. In addition, the abrogation phenomenon very likely represents a previously unknown "early" viral function, perhaps involving synthesis of a novel viral protein whose role in normal virus replication has yet to be determined.

Secondly, Fv-1 restriction represents a cellular control mechanism which is able to exhibit infection of specifiic MuLVs but not other, closely related strains. The relationship, if any, of this control mechanism to repression of endogenous viruses in mouse cells is not yet known, but the detailed knowledge of the specific biological and biochemical mechanism of Fv-1 restriction which is emerging will allow testing of other animal species for the presence of related control mechanisms.

Proposed Course:

The DNA synthesized in Fv-1 nonpermissive cells will be further analyzed, cloned, and sequenced, so that the mechanism responsible for its alteration can be deduced. Other restriction patterns, especially that in NIH cells will be analyzed by similar techniques. The series of studies using arrested and inhibited cells will be continued so that specific cellular requirements for synthesis of the mature forms of viral DNA can be studied.

Publications:

Bassin, R. H., Gerwin, B. I, Levin, J. G., Duran-Troise, G., Benjers, B. M., and Rein, A.: Macromolecular requirements for abrogation of Fv-1 restriction by murine leukemia viruses. J. Virol. August, 1980, in press.

Benade, L. E., Bassin, R. H., Ihle, J. N., Lee, J. C., and Rein, A.: Isolation and characterization of a unique C57BL B-tropic virus. Virology, 100: 199-203, 1980.

Benjers, B. M., Bassin, R. H., Rein, A., Gerwin, B. I., and Duran-Troise, G: Mechanism of restriction of murine leukemia viruses varies between different strains of Fv-1^n mice. Int. J. Cancer 24: 600-607, 1979.

Gerwin, B. I., Rein, A., Levin, J. G., Bassin, R. H., Benjers, B. M., Kashmiri, S. V. S., Hopkins, D., and O'Neill, B. J.: Mutant of B-tropic murine leukemia virus synthesizing an altered polymerase molecule. J. Virol. 31: 741-751, 1979.

Rein, A., Athan, E., Benjers, B. M., Bassin, R. H., Gerwin, B. I., and Slocum, D. R.: Isolation of a replication-defective murine leukaemia virus from cultured AKR leukaemia cells. Nature 282: 753-754, 1979.

Yang, W. K., Kiggans, J. O., Yang, D.-M., Ou, C.-Y., Tennant, R. W., Brown, A., and Bassin, R. H.: Synthesis and circularization of N- and B-tropic retroviral DNA in Fv-1 permissive and restrictive mouse cells. Proc. Natl. Acad. Sci. May, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04858-08-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Control of Growth and Differentiation of Hematopoietic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Molecular Virology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A biochemical assay has been developed for the src protein of Harvey sarcoma virus. The p21 src protein has been found to have a high affinity for guanine nucleotides but not adenine nucleotides. This assay, based on the binding of guanine nucleotides, provides a critical first step in elucidating a biochemical pathway for malignant transformation. A system for studying the mechanisms involved in erythroleukemia has been developed. Three distinct viruses can induce erythroleukemia in this system. Each virus causes a somewhat different form of erythroleukemia thus allowing for the first time the beginning of a biochemical basis for subclassifying a given form of cancer based on different biochemical mechanisms. This system and subclassification should have the same fundamental significance for cancer diagnosis as the ability to distinguish vitamin B12 vs iron deficient anemia did for non-malignant erythroid diseases some three to four decades ago.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The goal of this project is to define the biochemical pathways involved in (1) different forms of different cancers and (2) different forms of a given type of cancer. In the past, we have identified viral genes and gene products responsible for the induction of malignant transformation. Now we wish to discover how these gene products work and apply this knowledge to the development of new clinical tests for the differential diagnosis of different forms of human cancer.

Methods Employed:

A. Pulse labelling of cells with ^{35}S-methionine and immunoprecipitation using Staphylococcus aureus protein A. Analysis of precipitates on polyacrylamide slab gels. Fluorographic enhancement of protein bands. Tryptic peptide analyses using two-dimensional thin layer plates.

B. Restriction enzyme analyses of cloned and amplified sarcoma virus DNA. Southern transfer blotting assays and ethidium bromide containing cesium chloride gradients.

C. In vitro culture of bone marrow cells. Prolonged growth in cell culture of hematopoietic stem cells and granulocyte and erythroid precursor cells. Soft agar assays and methylcellulose assays for these cells. Histological identification of various hematopoietic cells.

D. Biochemical assays for phosphorylation and dephosphorylation of proteins. Gel electrophoresis, charcoal extractions, butanol two-phase extractions, thin layer chromatography.

E. Column chromatography using ion-exchange, hydroxiapatite, and hydrophobic column chromatography. Analysis of peptides by two-dimensional thin layer chromatography.

F. Gene transfer studies using liposomes, calcium phosphate precipitation and protoplasts, gene cloning using a variety of bacterial vectors.

Major Findings:

A. Last year a protein, p21, was identified as the transforming protein of Harvey and Kirsten sarcoma viruses. This is the first src protein, rigorously proved, to be identified as the transforming protein of a mammalian sarcoma virus. We have now developed a biochemical assay for this protein. p21

specifically binds guanine nucleotides and is phosphorylated by guanine nucleotides but not by adenine nucleotides. The biochemistry of this protein is being investigated in an effort to define a biochemical pathway that causes malignant transformation. The protein has been purified 300-fold from cellular extracts and its metabolism is being studied. The p21 thus is a unique protein, in that it is a GTP specific protein kinase. No other protein kinase specific for GTP has ever been described.

B. By injecting NFS Swiss mice with either Harvey virus, Friend virus or spleen focus-forming virus we can induce erythroleukemia. Harvey virus and Friend virus induce erythroleukemia and anemia. Harvey transformed cells are blocked at an earlier stage of erythroid differentiation than are the Friend transformed cells. The SFFV transformed cells are not blocked in differentiation but many more cells than normal are produced. The SFFV induced disease resembles polycythemia vera in humans. Thus, we have a system for defining a hierarchy of steps in over production of erythroid cells, i.e., erythroleukemia. This is analogous to the elucidation of the multiple ways that under production of erythroid cells in anemia can occur. The rational treatment of anemia could not occur before this classification was established. It is hoped that this system will lead to the same breakthrough in therapy of erythroleukemia and other forms of leukemia.

Significance to Biomedical Research and the Program of the Institute:

The fundamental problem in cancer research is to develop methods to subclassify cancer based on its etiology. It is known that cancer is a heterogeneous disease and that a given type of cancer can behave in unpredictable ways. The development of biochemical assays to subclassify cancers, based on the fundamental pathways involved in cancer causation, would represent the first critical step towards a new rationale approach to cancer therapy. A reasonable analogy is the causes of anemia. Before one could distinguish different causes of anemia there was no basis to decide whether to treat with iron or vitamins. No one would treat pernicious anemia with iron once it could be reliably distinguished from iron deficiency anemia. Similarly, the development of biochemical assays for transforming proteins allows the beginning of subclassification of cancers of apparently similar forms. This subclassification should provide the foundation for new approaches to the clinical diagnosis and therapy of human cancer.

Proposed Course:

The metabolism of p21 and GTP and GDP will be studied. The different steps in the p21 GTP cycle will be studied and assays developed for each step. The series of substrates and products of the pathway will be defined. This will lead to the elucidation of a biochemical pathway involved in the induction of malignant transformation. Markers will be developed for the different forms

of erythroleukemia and these markers will be correlated to the pathways affected by each virus which can induce that form of erythroleukemia.

Publications:

Scolnick, E. M., Papageorge, A. G., and Shih, T. Y.: Guanine nucleotide-binding activity as an assay for src protein of rat-derived murine sarcoma viruses. Proc. Natl. Acad. Sci. USA 76: 5355-5359, 1979.

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Shih, T. Y., and Scolnick, E. M.: The molecular biology of mammalian sarcoma viruses. In G. Klein (Ed.): Viral Oncology. New York, Raven Press, 1980, in press.

Scolnick, E. M., Oliff, A., Linemeyer, D., Ruscetti, S.: Molecular genetics and cell culture assays for helper-independent and repliction-defective components of the Friend virus complex. In Fields, B., Jaenisch, R., Fox, C. F. (Eds.): Animal Virus Genetics. New York, Alan R. Liss, Inc., 1980, in press.

Honors and Awards

Eli Lilly Award for Microbiology and Immunology - 1980

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04899-08-LTVG

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Transcription and Translation of Avian Oncorna Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

AMV virus is a mixture of three viruses: a leukemogenic agent and two myeloblastosis associated helper viruses, MAV-1 and MAV-2. The ratio of the helpers to AMV is about 10:1. The genomic size of the two helpers was determined to be 7500 basis and that of AMV 7000. Complete DNA duplexes of genomic RNAs have been synthesized in vitro. The duplexes were estimated to be 5.2 and 4.0 x 10^6 daltons for helper and AMV respectively. Duplex AMV fragments synthesized in vitro were inserted into pBR322 and cloned in an approved EK2 host. 28 clones containing AMV inserts were isolated. These clones represent specific segments of the viral genome. cDNA probes prepared from AMV (MAV) were hybridized to a Southern blot restriction fragment for cell line Q5 DNA. This line consists of quail cells that have been transformed by MC29 but do not produce virus. The probe hybridized predominately to a single Bam HI fragment and a single Eco RI fragment which suggests that the integrated provirus within Q5 is located at a single site that is stable through cell division.

Project Description

Objectives:

The scope of this investigation is to delineate the relationship between virus gene expression and conversion of cells from normal to malignant state and to study the molecular anatomy of known tumor viruses and describe the mechanism by which subviral structures act in concert with cellular factors to regulate oncogenesis. The technique of molecular cloning, DNA sequence analysis, and site specific mutagenesis will be used to implicate specific nucleotides in the transformation process.

Methods Employed:

A. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by *in vitro* packaging. Isolation of phage of libraries containing virus related sequences by hybridization of cDNA probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure.

B. Construction of Recombinant Plasmids. Double stranded cDNA of AMV genome was prepared. It was converted to perfect duplex molecules with blunt ends by digestion with single-strand nuclease S_1. It was then incubated with *E. coli*. DNA polymerase I. The product of the S_1-DNA polymerase reaction was ligated to Bam HI linkers with T4 DNA ligase. pBR322 was digested with Bam HI, treated with alkaline phosphotase. It was then ligated to double stranded cDNA linked to Bam HI linkers.

C. Transformation and Identification of Recombinant Clones. Construction of chimeric plasmids and the transformation of *E. coli*. X1776 by these plasmids was performed in a p2 physical containment laboratory. X1776 was transformed by a transfection procedure. Transformed colonies containing AMV sequences were identified by colony hybridzation. The colonies were screened with ^{32}P-labeled AMV cDNA. Strongly hybridized colonies were selected and replated, single colonies were picked and grown.

Major Findings:

A. A DNA endonuclease, Endo-I, which cleaves superhelical DNAs, has been isolated from avian myeloblastosis virions stripped of their coats by mild detergent treatment. The enzyme has a broad pH optimum around 7.5-8.0 and requires Mg^{2+} for activity. A second endonuclease, Endo-II, with a requirement for Mn^{2+}, also present in viral cores, copurified with avian myeloblastosis virus $\alpha\beta$ DNA polymerase (reverse transcriptase, RNA-dependent DNA

nucleotidyltransferase) and similarly cleaved superhelical DNAs. Heat denaturation and sodium fluoride and N-ethylmaleimide inhibition studies were carried out to demonstrate a possible relationship between the two endonucleases and the viral DNA polymerase and RNase H activities. It appears that Endo-II may be an intrinsic activity of the polymerase.

B. Avian DNA polymerases use host $tRNA^{Trp}$ as the primer for transcription. Bovine $tRNA^{Trp}$ has been previously shown to be a biologic stubstitute for the avian primer. A bovine $tRNA^{Trp}$ fragment has been identified as having a high binding affinity for the polymerase. The fragment is assigned to be 67 nucleotides, and contains most of the elements required to maintain the secondary and tertiary structure of $tRNA^{Trp}$.

C. A native ribonucleoprotein (RNP) complex of avian myeloblastosis virus was prepared under conditions that gave optimal cDNA synthesis. The complex was an autonomous transcriptional unit capable of synthesizing DNA complementary to the RNA virus genome in the absence of exogenous reverse transcriptase (RNA-dependent DNA nucleotidyltransferase), genomic RNA, and primer. The RNA of the RNP complex cannot be translated in an in vitro cell-free translational system. The RNP contains intact viral RNA, the two subunits of the reverse transcriptase (β and α), the p32 polypeptide resulting from the cleavage of the β subunit into the α subunit, and p12. The principal polypeptide constituent of the RNP complex is the highly basic protein p12, which occurs at a molar ratio of 40:1 in relation to the β subunit of the polymerase. When examined by the electron microscope, the RNP complex appears similar to the beaded structure of chromatin fiber. A significant portion of these molecules are circular, with headlike structures attached. The circular nature of the proviral DNA and the ability of the RNP complex to generate large intact cDNA copies from the natural primer end suggest that the 5' and 3' ends of the viral RNA are in proximity when in the RNP complex.

D. Rate sedimentation of sheared chromatin from chick embryo fibroblasts (CEF) fractionated on glycerol gradients yields two chromatin fractions, a heavy (or fast sedimenting) component, and a light (or slowly sedimenting) component. cDNA prepared from avian myeloblastosis virus (AMV) hybridizes to equal extent with both the DNA moieties extracted from light and heavy chromatin fractions, indicating that the sequences hybridizing to AMV-cDNA are equally distributed between light and heavy fractions. Consequently, it is concluded that the endogenous proviral sequences are randonly integrated in the chromatin of CEF. However, when CEF transformed by Rous sarcoma virus (RSV), which shares common sequences with AMV, molecular hybridization revealed that most of the exogenous proviral sequences were integrated within the light chromatin fraction. The results suggest that exogenous proviral sequences integrate in chromatin regions of particular configuration. Indeed, the light chromatin fraction, i.e., the chromatin fraction enriched in exogenous proviral sequences, as opposed to heavy fraction, was found: a) enriched in low temperature melting material; b) more accessible to digestion by DNase I; and, c) better template for RNA synthesis in the presence of RNA

polymerase. In addition, the chromatin fraction enriched in proviral sequences exhibits qualitative and quantitative differences in the protein-moiety.

E. AMV virus is a mixture of three viruses: a defective leukomogenic agent and two myeloblastopic associated helper viruses; MAY-1 and MAY-2. The ratio of the helpers to AMV is about 10:1. Denaturing gels indicate that the genomic size of the two helpers is 7500 bases and that of the AMV 7000. Complete DNA duplexes of genomic RNA's have been synthesized in vitro utilizing AMV DNA polymerase. The duplexes were estimated to be 5.2 and 4.0×10^6 daltons for helper and AMV respectively. Duplexes were electro-eluted from agarose gels and subjected to restriction enzyme mapping.

F. Duplex AMV fragments synthesized in vitro were inserted into pBR322 and cloned in an approved EK2 host. 28 phonotypically correct clones containing AMV inserts were isolated. These clones represent specific segments of the viral genome. Clone T8 with an insert of 680 bases, has been analyzed by restriction endonuclease digestion and represent the 3'-end of the AMV genome.

G. cDNA probe prepared from an AMV(MAV) RNA template was hybridized to a southern blot restriction fragments for cell line Q5 DNA. This line consists of quail cells that have been transformed by avian myelocytomatosis virus MC29, but do not produce virus. The probe hybridized predominately to a single Bam H1 fragment and a single Eco RI fragment which suggests that the integrated provirus within Q5 is located at a single site that is stable through cell division. The probe used in this experiment was shown to be virus specific since it predominately hybridized to restriction fragments in chicken DNA that have been found to contain endogenous virus related sequences.

Significance to Biomedical Research and the Program of the Institute:

It is clear that the formation, the maintenance and the expression of provirus are the central features of the life cycle of RNA tumor viruses. Many questions related to these aspects are unsettled. To elucidate the process of oncogenesis induced by these viruses it is important to understand the structural organization of viral genes within the host chromosome and the process by which these genes are expressed and regulated.

Proposed Course:

Current research is conducted toward defining the mechanism of action of malignant transformation in cells. Efforts are concentrated towards:

1. Analysis of transformation sequences specific for AMV and non-homologous to MAV. The 28 clones will be fully characterized by restriction enzyme analysis combined with DNA transformation.

2. Analysis of viral expression in different cell types--restriction for replication of the transforming virus in non-target cells (e.g., fibroblasts). Analysis of AMV specific RNA synthesis in target cells. Maniotis' modified northern blot technique will be utilized in these experiments.

3. AMV specific clones will be utilized to R-loop AMV specific subgenomic RNAs in leukemic myeloblasts. These RNAs will be in turn translated into leukemic specific polypeptides.

4. We have successfully made antisera against the "src" protein (in marmosets) of RSV. These antisera will be utilized to further study some of the properties of the transforming protein.

5. Phage λ derivatives containing the MC29 proviral sequence will be isolated from libraries prepared from partially disected or size fractionated completely digested DNA from MC29-transformed quail cell line Q5. We will determine if mouse 3T3 cells can be transformed by the proviral MC29 sequence within Q5. It is very likely that this will be possible since Copland and Cooper have been able to transform these cells with DNA from an MC29-transformed mouse nonproducer line. A survey will be made with restriction enzymes that do not destroy transformation activity and thus do not cleave within the sequence required for transformation. Sub-genomic segments from the cloned DNA will be tested for transforming ability. This should define specific sequences required for transformation. Interesting portions of the recombinant phages required for transformation will be sequenced. Mutants in these sequences will be isolated by the site specific mutagenesis techniques. These mutants will be assayed for transformation ability in order to implicate specific nucleotides in the transformation process.

Publications:

Samuel, K. P., Papas, T. S., and Chirikjian, J. G.: DNA endonuclease associated with avian myeloblastosis virus DNA polymerase. Proc. Natl. Acad. Sci. USA 76: 2659-2663, 1979.

Chen, M.-J., and Papas, T. S.: Native ribonucleoprotein is an efficient transcriptional complex of avian myeloblastosis virus (AMV). Proc. Natl. Acad. Sci. USA 77: 1296-1300, 1980.

Schulz, R. A., Chirikjian, J. G., and Papas, T. S.: Avian myeloblastosis virus (AMV) linear duplex DNA: In vitro enzymatic synthesis. In Russell, T. R. and Brew, K. (Eds.): From Gene to Protein, Information Transfer in Normal and Abnormal Cells. New York, Academic Press, 1979, pp. 624.

Schulz, R. A., Chirikjian, J. G., and Papas, T. A.: In vitro enzymatic synthesis and structure analysis of RNA tumor virus proviral DNA. XIth International Congress of Biochemistry. p. 37, 1979.

Papas, T. S., Chen, M.-J., and Chirikjian, J. G.: Transcriptional Complexes of Avian Myeloblastosis Virus (AMV). In Young, D. S. (Ed.): Advances in Comparative Leukemia Research. Elsevier-North Holland, 1979 (in press).

Chirikjian, J. G., and Papas, T. S.: Structural Specificity of the Interaction of $tRNA^{Trp}$ (bovine) to AMV DNA Polymerase. In Young, D.S. (Ed.): Advances in Comparative Leukemia Research. Elsevier-North Holland, 1979 (in press).

Pantazis, P., Schultz, R. A., and Papas, T. S.: Distribution of Proviral Sequences in Chromatin of Uninfected and Rous Sarcoma Virus Infected Embryonic Fibroblasts. (in press).

Schulz, R. A., Pantazis, P., and Papas, T. S.: Integration and Expression of Rous Sarcoma Virus DNA in Transformed Chick Embryo Fibroblasts. In Scott et al. (Eds.): Mobilization and Reassembly of Genetic Information. New York, Academic Press, 1980 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04963-05-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Studies on the Genome and Gene Products of Kirsten and Harvey Murine Sarcoma Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	H. Langbeheim	Visiting Fellow	LTVG, NCI
	R. Ellis	Staff Fellow	LTVG, NCI

COOPERATING UNITS (if any)

M. Willingham and I. Pastan	LMB, NCI
E. Chang and D. Lowy	DBCD, NCI
M. C. Lin	LNE, NIAMDD

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Molecular Virology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	3.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project is currently directed toward characterization and purification of the p21 src protein coded for by the rat-derived RNA sarcoma viruses including Harvey and Kirsten strains of murine sarcoma viruses and a rat sarcoma virus of pure rat origin. The fact that p21 is indeed the transforming src protein of these viruses has been established by study of this protein (1) in a Ki-MSV mutant (ts371) temperature-sensitive for the maintenance of transformation and (2) in cells transformed by molecularly cloned Ha-MSV DNA. An enzymatic activity catalyzing a GTP-specific autophosphorylation of the p21 src protein has been found in the purified p21 protein. An endogenous p21 homologous to the viral p21 src has been found in many normal vertebrate cells including human. The p21 src has been localized to the inner surface of the plasma membrane of virus-transformed cells suggesting such a cellular location the major site of action of this transforming protein.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The scope of this project is to study the molecular virology of Kirsten (Ki-MSV) and Harvey (Ha-MSV) strains of murine sarcoma viruses. The major objectives are threefold: (1) to delineate the genomic structure of these two viruses; (2) to identify the viral gene products; and (3) to study the mechanisms through which these viruses transform normal cells.

Both Ki-MSV and Ha-MSV are fibroblast-transforming and sarcoma-inducing viruses isolated by passages of murine type-C helper viruses through rats. Both are recombinant viruses with rat genetic sequences as large inserts between sequences derived from the helper type-C viruses at both ends of the viral genomic RNA. Therefore, they are excellent model systems to study mechanisms of cell transformation directly caused by the viral gene products and to explore the role of the normal host genetic information and gene products in the oncogenic potential of these recombinant transforming viruses.

Methods Employed:

(1) The protein products coded for by the viral genomes were identified by employing antisera containing antibodies directed against virus gene products from young Osborne-Mendel rats bearing tumors induced by syngeneic transplantation of the nonproducer cells transformed by these viruses. Cellular proteins were labeled by growing cells in medium containing [^{35}S] methionine. After immune precipitation with antisera, the precipitated proteins were analyzed by the SDS-polyacrylamide gel electrophoresis.

(2) The proteins coded by Ki-MSV and the Ha-MSV were purified on a large scale from virus-transformed cells by a [^{3}H]GDP-binding assay specific to p21. Cellular extracts incubated with [^{3}H]GDP were immunoprecipitated with anti-p21 antisera. After washings, the [^{3}H]GDP bound to p21 in the immunoprecipitates was counted in a liquid scintillation counter. Ha-MSV p21 of NIH 3T3 cells transformed by Ha-MSV has been purified from cell extracts through hydroxyapatite chromatography and hydrophobic chromatography involving phenyl-Sepharose.

(3) The enzymatic activities associated with the purified p21 were studied by the standard methods of enzymology in a soluble reaction mixture. The phospho-transferase activities were studied by the transfer of ^{32}P-labelled at the gamma-phosphate of the nucleotide triphosphates. The phosphorylated proteins labelled with [^{32}P] were examined by SDS-polyacrylamide gel electrophoresis.

Major Findings:

(1) The p21 coded for by the Ha-MSV or Ki-MSV is the src gene product. The identification of the p21 coded for by Ha-MSV/Ki-MSV prompted us to establish whether or not this is indeed the src gene product of these sarcoma viruses responsible for the virus-induced cell transformation. Several lines of evidence gathered so far firmly established that p21 is indeed the transforming protein. First of all, the p21 coded for by a temperature-sensitive mutant of Ki-MSV (ts 371) is very thermal-labile as compared to the p21 of the wild-type virus, indicating the thermal lability of the ts p21 is the lesion responsible for the heat-sensitive lesion in the ts mutant. Secondly, through transfection studies of the molecularly cloned Ha-MSV and its subgenomic fragments, the transforming src gene of Ha-MSV has been localized to the 1100 nucleotide sequences closest to the 5' end of the viral genome. We have found that this is also the coding region for the p21 protein. Therefore, p21 is the transforming protein responsible for transformation of NIH 3T3 cells by these molecularly cloned Ha-MSV DNA.

(2) The p21 src of Ha-MSV possesses enzymatic activity of GTP-specific autophosphorylation. p21 is a specific guanine nucleotide-binding protein. By examining a variety of nucleotides for the stabilization of the thermal lability of the ts Ki-MSV p21, we found that only GTP, GDP and dGTP have the ability to stabilize the ts p21, indicating that p21 is a protein having affinity for these guanine nucleotides. Based on this property, a [^{3}H]GDP-binding assay specific to p21 has been developed. This convenient and rapid assay permitted us to large-scale purify the viral p21 from the NIH 3T3 cells transformed by Ha-MSV for detailed biochemical study of this src protein. Employing several purification steps involving hydroxyapatite chromatography and hydrophobic column of phenyl-sepharose, we have purifed this protein approximately 300-fold from cell lysates. We found an enzymatic activity of the purified p21 for GTP-specific autophosphorylation. Consistent with the guanine nucleotide binding activity of this protein, [γ-^{32}P]GTP and [γ-^{32}P]dGTP can serve as donors for p21 phosphorylation. No such activity is found for [γ-^{32}P]ATP or [γ-^{32}P]dATP, common substrates for most of the protein kinases. No phosphorylation of other proteins except p21 has been detected. Therefore, p21 is a very unusual phospho-transferase catalyzing GTP/dGTP specific autophosphorylation.

(3) Cellular localization of the p21 protein. Using electron microscopic immunocytochemistry, we have investigated the intracellular location of the p21 src protein in cells transformed by Ha-MSV. The distribution of p21 in intracellular sites in MDCK dog cells transformed by Ha-MSV was examined and quantified. Over 95% of p21 was localized to the inner surface of the plasma membrane in these Ha-MSV transformed cells; p21 was not exposed on the outside surface of the plasma membrane. The cellular location of p21 is similar to that of the p60src of avian sarcoma viruses suggesting that the plasma membrane is a major site of action for transforming proteins.

(4) Identification of a normal vertebrate cell protein related to the p21 src of Ha-MSV. Using tumor-bearing rat sera containing antibodies directed against Ha-MSV p21, we have detected lower amounts of a 21,000 dalton cellular homologue of the viral p21 in uninfected cells derived from a variety of vertebrate species including rat, mouse, mink, hamster, rabbit, turkey, bat, cat, dog, horse, monkey and human. This endogenous p21 shares cross-immuno-reactivity and peptide maps with the Ha-MSV p21. The endogenous p21 is itself a structurally highly conserved protein in a variety of vertebrate species. The present results suggest that a normal cellular gene encoding an antigenically and chemically related protein of the Ha-MSV p21 is present in many vertebrate species including human.

(5) Selective loss and restoration of peptide hormone receptors in an established cell line (MDCK). Using an MDCK cell line transformed by Ha-MSV, we found peptide hormone receptors such as glucagon receptor are selectively lost or reduced in this cell line as compared to the parental MDCK. Sodium butyrate-treatment of the transformed cells partially restores the sensitivity of adenylate cyclase to glucagon and vasopressin to that seen in the parent cells.

Significance to Biomedical Research and the Program of the Institute:

The virus-induced transformation of normal target cells has long been theorized to be mediated by transforming proteins coded for by these transforming viruses. In the rat-derived RNA sarcoma viruses including Kirsten and Harvey strains of murine sarcoma viruses and a rat sarcoma virus, we have found such a transforming src protein. Through the biochemical study of this src protein, and studies on its cellular function, eventually we should be able to elucidate the molecular mechanisms through which these sarcoma viruses transform the normal target cells. The identification of a normal cell homologue of this viral src protein in a variety of vertebrate species including human suggests and confirms a widely held theory that all normal vertebrate cells harbor several genes encoding proteins homologous to the transforming proteins of several RNA sarcoma viruses such as the rat-derived RNA sarcoma viruses, avian sarcoma viruses and perhaps the Abelson murine leukemia virus. A central question in the origin of the transforming genes of various RNA tumor viruses and the function of their normal cellular homologues in normal animal species including human is whether the src proteins of the sarcoma viruses is qualitatively different from their cellular homologues or they are simply quantitatively overproduced in the virus-transformed cells through recombination of the cellular transforming genes with the virus genomes. If viral src genes are mutations of normal cellular sarc genes, then we can ask the question whether these genes are the targets of chemical carcinogens, most of them are mutagens. If the viral src proteins are simply overproduced in the virus-infected cells resulting in cell transformation, then we can ask the question of what is the gene regulation mechanisms normally control the expression of these

genes in healthy cells. Through the study of viral transforming proteins and their normal cellular homologues, we shall be able to shed light on these questions and to understand the fundamental problem of the mechanisms of human malignancy.

Proposed Course:

Efforts will be made to further characterize the biochemical activities of the purified Ha-MSV p21 src protein and to study its cellular function related to virus-induced cell transformation. The endogenous p21 present in several vertebrate species including human will be further characterized to determine whether its function is similar to the viral src protein or the viral src protein is a mutated form of the endogenous normal cellular sarc protein.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04970-04-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemistry of Cellular Transformation by Avian Sarcoma Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. P. Bader	Head, Cellular Transformation Sec.	LTVG, NCI
OTHER:	L. S. W. Lee	Visiting Fellow	LTVG, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION Cellular Transformation Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.5	2.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Malignant transformation by the avian Rous Sarcoma Virus (RSV) is induced by a virus-coded protein which plays no role in virus reproduction. Attempts have been made to identify the transforming protein of the Bryan "high titer" strain of RSV (RSV-BH) and to determine its biochemical function in transformation. Characteristic morphological and metabolic features of RSV-BH transformed cells have been used as a basis for examining possible functions of the transforming protein, and a mutant of RSV-BH which induces temperature-dependent transformation has been useful in distinguishing between direct and indirect effects of the protein. Mammalian cells from several species have been transformed by RSV-BH, and these are being used to analyze viral transformation in the absence of inherent complicating features of avian host cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To identify the virus-coded protein responsible for the malignant transformation of cells by Rous sarcoma virus; to determine the biochemical function of this protein; to determine the primary physiological effects resulting from the functioning of this protein; to describe the sequence of metabolic changes which result in the altered metabolic profile characteristic of malignant cells; to distinguish metabolic changes necessary to the maintenance of the malignant state from those which are irrelevant.

Methods Employed:

(A) Transformation of cells in culture by avian and murine sarcoma viruses, selection of mammalian cells transformed by avian viruses, morphological resolution of transformed cells by microscopy, resolution of cellular organelles by supravital staining.

(B) Isolation of cellular organelles, including microsomes, cell surface membranes, and nuclei.

(C) Quantitative chemical determinations of protein, and a variety of enzymes, and qualitative resolution of isozymes of about 30 different enzymes.

(D) Complement fixation, immunoprecipitation, immuno-affinity chromatography.

(E) Dextran and sucrose density gradients, polyacrylamide gel electrophoresis, starch gel electrophoresis, autoradiography, fluorography.

(F) Uptake of radioactive molecules into cells, incorporation of radioactive precursors into macromolecules, binding of radioactive molecules to specific cell-surface sites.

(G) Analysis of chromosomes by staining and banding.

Major Findings:

Attempts have been made to identify the virus-coded protein responsible for the malignant transformation induced by Rous sarcoma virus (RSV), and to determine the function of this protein. The Bryan "high titer" strain of RSV (RSV-BH) induces a unique and characteristic morphological change in infected cells. A mutant of this virus (RSV-BH-Ta), isolated in this laboratory, induces temperature-dependent transformation. At 41° infected cells appear nontransformed, but after shifting to 37°, a variety of morphological

and biochemical changes occur, resulting in a transformed phenotype indistinguishable from cells transformed by wild type RSV-BH. We have identified several metabolic changes induced during transformation which require new RNA synthesis and protein synthesis, including increased glucose uptake and glycolysis, increased hyaluronic acid synthesis, decreased alkaline phosphotase activity, and decreased adenosine deaminase activity. These metabolic changes are preceded by morphlogical changes characteristic of RSV-BH transformation which occur even while RNA synthesis or protein synthesis is inhibited.

The observation of an increase in intracellular water in RSV-BH transformed cells suggested that ionic concentrations may be altered also. The potential significance of ionic changes in carcinogenesis is suggested in changes in transcription and translation observed in several model systems. The rate of uptake of Na^+, as well as the intracellular concentration of Na^+, was found to be substantially higher in RSV-BH transformed cells than in nontransformed cells, or in cells transformed by a related virus. A smaller increase in the rate of K^+ uptake was observed, and intracellular K^+ concentrations were similar to nontransformed cells. Nonetheless, the increased rate of Na^+ uptake apparently is related to an induced effect of the RSV-BH transforming protein on cellular transcription, and is not a direct function of the viral protein. Inhibition of RNA synthesis (or protein synthesis) prevents the increase in Na^+ uptake rate which occurs after shifting RSV-BH-Ta infected cells from the nontransforming temperature (41°) to the transforming temperature (37°), conditions which should allow the synthesis of functional transforming protein.

The increased uptake of Na^+ in RSV-BH transformed cells helps to explain another phenomenon characteristic of these cells, the nonrepressibility of the glucose uptake system. In nontransformed cells the capacity for glucose uptake decreases after incubation with increasing concentrations of glucose in the culture medium. In RSV-BH transformed cells, the glucose uptake capacity usually is 2 to 5 times that of nontransformed cells, and is unaffected by variations in glucose concentrations. We have found that intracellular ATP levels are negatively correlated with glucose uptake capacity in nontransformed cells, and that experimentally increasing Na^+ uptake decreases intracellular ATP and increases glucose uptake capacity. In RSV-BH cells ATP levels were found to be about half that of nontransformed cells, consistent with the observed higher Na^+ uptake of transformed cells, and with the greater capacity for glucose uptake.

A common feature of cells transformed by RSV-BH is the occurrence of apparently multinucleated cells, or cells in which nuclei are fragmented into many small nuclei. The proportion of cells with such nuclear abberations increases after exposure to 2-deoxyglucose, or a variety of other compounds known to interfere with glycosylation reactions (glucosamine, tunicamycin, etc.). Such treatments can result in over half of the RSV-BH transformed cells in a culture containing multinuclei, while identical treatments of nontransformed cells results in less than 1% multinucleated cells. Cells of several species

transformed by RSV-BH undergo this nuclear change, while cells transformed by other viruses are relatively unaffected. Induced multinucleation is not lethal, and cells proceeding through mitosis return to the nonnucleated state. A glycosylated molecule of nontransformed cells, which is absent or underglycosylated in RSV-BH transformed cells, has been identified, and its role in maintaining nuclear integrity is under investigation.

Attempts to develop an antiserum agains the RSV-BH coded transformation protein have not yet succeeded. Animals (chicken, turkeys, rabbits, and rats) with developing RSV-BH tumors have yielded a variety of sera with antibodies against one or more structural proteins of the virion, but none which consistently select a virus-specific nonstructural protein in immunoprecipitation or immunoaffinity chromatography systems. The inability to obtain antibody readily against RSV-BH transforming protein may be an indication of common structural similarities between the transforming protein and a normal cellular protein.

The transformation of mammalian cells by the avian RSV-BH has allowed us to obtain a variety of cells containing the defective RSV-BH genome, devoid of helper virus which normally accompanies RSV-BH. Non-virus-producing hamster clones readily yield infectious virus when hybrids are formed with avian cells producing a helper virus. This rescue occurs within a few hours after hybrid formation, and new DNA synthesis is not required, demonstrating that the hamster cells synthesize full genome copies of viral RNA. Coalescence of hamster mininuclei with mouse cells resulted in the establishment in mouse cells of identifiable hamster chromosomes containing functional RSV-BH genome. The rescuability of viral genome from hybrid cells is under investigation. Many attempts to establish hamster or rat chromosomes in avian cells, by cellular hybridizations, nuclear transfers, or direct chromosome transfers, were unsuccessful, suggesting that rodent chromosomes may not reproduce in avian cells.

Significance to Biomedical Research and the Program of the Institute:

The identification of a single protein responsible for malignant transformation, and resolution of that protein's function, would be major advances in our understanding of the nature of cancer cells. Recognition of a primary physiological imposition on the cell which converts that cell to a malignant form, and the identification of characteristic metabolic changes induced during the development of transformation, would allow a description of the entire sequence of metabolic events which culminate in malignancy. Such studies could lead to a determination of metabolic events necessary to the maintenance of the malignant state, but not to normal metabolism, and the possibility of therapeutic intervention in such metabolism.

Proposed Course:

To characterize RSV-BH specific protein, to determine the primary function responsible for malignancy, and to describe the sequence of metabolic changes culminating in the phenotype characteristic of RSV-BH transformed cells. Also, development of gene transfer techniques for inquiries into the localization and regulation of transforming viral genes.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04980-03-LTVG

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Proteins Associated with the Biological Effects of Type-C Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH Carcinogenesis Intramural Program
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SECTION
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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The proteins encoded by several type-C retroviruses have been studied. Both polycythemia- and anemia-inducing strains of defective spleen focus-forming virus (SFFV) code for 52,000 dalton glycoproteins which share determinants with the gp70's of MCF viruses. Studies using molecularly cloned SFFV DNA indicate that the expression of gp52 is correlated with the biological effects of SFFV. The helper-independent Friend murine leukemia virus (F-MuLV) induces an erythroleukemia-like disease in certain strains of newborn mice that is associated with the expression of an MCF-like virus. Susceptibility to this disease appears to be under control of genes distinct from the Fv-2 gene controlling susceptibility to SFFV. Studies with a subgenomic fragment of molecularly cloned F-MuLV DNA suggest that the env gene of F-MuLV, and by extrapolation its gene product, is associated with disease induction. Studies are in progress to determine the role that proteins encoded by SFFV and F-MuLV play in diseases induced by these viruses. In other studies, 3 isolates of feline sarcoma virus (FeSV) have been shown to code for distinct gag-x polyproteins. Studies are currently being carried out to determine the role that these proteins play in FeSV-induced transformation.

Project Description

Objectives:

(1) To characterize proteins associated with the erythroleukemia-like diseases induced by either the defective spleen focus-forming virus (SFFV) or the helper-independent Friend murine leukemia virus (F-MuLV) and to determine the role that these proteins may play in disease induction.

(2) To characterize proteins encoded by independent isolates of the feline sarcoma virus (FeSV) and their transformation-defective mutants and to determine the role that these proteins may play in transformation.

Methods Employed:

(1) Preparation of specific antisera to viral-encoded proteins, including the preparation of monoclonal antibodies.

(2) Use of specific antisera in competition radioimmunoassays to detect viral-encoded proteins in various virus-infected cells and tissues.

(3) Use of specific antisera to precipitate proteins from cytoplasmic extracts of metabolically labeled virus-infected cells and tissues.

(4) Analysis of immune precipitates by SDS-polyacrylamide gel electrophoresis and autoradiography.

(5) Analysis of viral protein expression in individual cells by immunofluorescence.

Major Findings:

(A) Studies on SFFV and F-MuLV:

(1) All polycythemia-inducing strains of the spleen focus-forming virus ($SFFV_{FV-P}$) code for 50-52,000 dalton glycoproteins which share determinants with the gp70s of MCF viruses. In addition, certain strains of $SFFV_{FV-P}$ code for a 45,000 dalton gag-related protein. In order to compare the proteins encoded by polycythemia-inducing strains of SFFV with anemia-inducing strains of SFFV ($SFFV_{FV-A}$), cells nonproductively infected with the later virus were subjected to the same analyses previously carried out on cells nonproductively infected with the polycythemia-inducing strains of SFFV. It was shown that $SFFV_{FF-A}$, like all isolates of $SFFV_{FV-P}$, codes for a 52,000 dalton glycoprotein which shares determinants with the gp70s of MCF viruses. This virus also encodes a gag-related protein of 45,000 daltons. Thus, SFFVs derived from FV-P or FV-A stocks code for closely related proteins.

(2) Cells transfected with molecularly cloned SFFV DNA, either the entire genome or certain subgenomic fragments, were shown to express the gp52 encoded by SFFV. When SFFV is rescued from these cells and injected into susceptible mice, it will induce disease as well as the synthesis of gp52.

(3) Additional studies have been carried out on the gag-related protein coded for by the Lilly-Steeves strain of SFFV. The primary translational product is a 45,000 dalton protein that contains determinants of F-MuLV p12 as well as some p30 determinants. Both a phosphorylated and a glycosylated form of the p45 can be detected in cells nonproductively infected with the Lilly-Steeves strain of SFFV as well as a 65K form that contains terminal sugars. In studies carried out in collaboration with Drs. R. Steeves and R. Anand of Albert Einstein College of Medicine, New York, it was shown that the gag-related protein of SFFV can enter specifically into the envelope of SFFV RNA-containing virions after pseudotyping with helper-virus.

(4) Cells transfected with a subgenomic fragment of molecularly cloned F-MuLV were shown to express the env gene product of F-MuLV but not the gag gene product. Helper-independent viruses cloned after the above cells were rescued with the amphotropic retrovirus 1504A included a recombinant virus which codes for the gag gene product of the amphotropic virus and the env gene product of F-MuLV.

(5) Molecularly cloned F-MuLV induces in newborn mice an erythroleukemia-like disease characterized by splenomegaly and severe anemia. When various strains of mice were analyzed for susceptibility to this disease, it was found that certain strains were susceptible, such as NIH Swiss and BALB/c, while other strains, such as DBA/2 and C57BL/6, were resistant. Since DBA/2 mice are Fv-2^s, susceptibility to the F-MuLV-induced disease does not appear to be under control of the Fv-2 gene, which governs susceptibility to SFFV-induced disease. Preliminary studies indicate that resistance to F-MuLV-induced disease is dominant. Untreated adult NIH Swiss mice are resistant to the F-MuLV-induced disease, but can be made susceptible by such pretreatments as x-irradiation, phenylhydrazine, bleeding and silica. When spleen homogenates from the various mice were passaged on fibroblasts and then analyzed for viral protein expression, MCF-specific gp70 could be detected in cells infected with spleen homogenates from all susceptible animals (NIH Swiss and BALB/c newborns as well as pre-treated adults) but not from resistant mice (including untreated NIH Swiss adults), although ecotropic viral proteins were detected in all spleen homogenates. In contrast to results obtained from F-MuLV infected animals, an MCF-like gp70 could be detected in bone marrow cells from uninfected mice that were resistant to the F-MuLV-induced disease but not in the marrow of mice susceptible to the disease.

(6) Studies were carried out in collaboration with Dr. Michael Dexter, Paterson Laboratories, Manchester, England, to characterize the proteins being synthesized in a long term bone marrow culture, designated 416B, established after F-MuLV/SFFV infection. It was shown that this culture

expressed an ecotropic F-MuLV that coded for an env-gene precursor protein that was 5000 daltons smaller in molecular weight than that of 4 other stocks of F-MuLV. The culture, however, did not express the marker protein for SFFV, gp52. It was concluded that the continuous growth of this culture as a "stem cell" line cannot be attributed to the continued expression of SFFV, as originally suggested.

(B) Studies on FeSV

The following studies were carried out in collaboration with C. J. Sherr, L. Turek and L. Donner, Laboratory of Viral Carcinogenesis, NCI:

(1) Cells nonproductively transformed by the Snyder-Theilen, Gardner-Arnstein and McDonough strains of FeSV were shown to synthesize gag-x polyproteins of 78,000, 100,000 and 180,000 daltons, respectively. These FeSV-encoded products can be precipitated by antisera to polypeptides encoded by the gag gene of FeLV and by rat antisera raised to FeSV-transformed rat tumor cells. Precipitation with rat antisera absorbed with FeLV showed that the x portions of the three gag-x proteins are each antigenically distinct, suggesting that the src genes of the three independent isolates are not identical. Anti-x sera failed to precipitate products from radiolabeled cat lymphoid tumor cells (FL74) and therefore lack reactivity to the FeLV-induced tumor specific antigen, FOCMA.

(2) Mink cells infected with transformation defective mutants of the ST-strain of FeSV are flat, do not form tumors in nude mice, yet they contain FeSV provirus integrated at the same molecular site as cells infected with their transforming counterparts and express viral RNA. In order to determine if viral proteins were being expressed in these cells, various immunoassays were performed. The phenotypically normal cells were shown to express the phosphorylated gag-x polyprotein encoded by gag and src sequences of the FeSV genome at levels approaching that of the transformed cells.

Significance to Biomedical Research and the Program of the Institute:

Understanding the proteins that are responsible for the biological effects of leukemia and sarcoma viruses is of great importance. The erythro-leukemia-like diseases induced in mice by the spleen focus-forming virus and the Friend leukemia virus are associated with the expression of specific viral proteins which are products of an env gene of each virus. Transformation of fibroblasts in vitro by independent isolates of the feline sarcoma virus is associated with the expression of a specific FeSV-encoded protein that is a product of a fused gag and src gene of each virus. It is hoped that through further characterization of the proteins encoded by these murine and feline viruses a correlation between biochemical parameters and biological effects will be found. Using reagents and information gained from studying these model systems may ultimately help us to develop immunoassays which would detect cross-reacting leukemia- or sarcoma-specific proteins in primate species such as man.

Proposed Course:

(A) Studies on SFFV and F-MuLV

(1) The gp52 from rat cells nonproductively infected with the spleen focus-forming virus will be further purified using affinity and ion-exchange chromatography as well as gel filtration. Monoclonal antibodies to gp52 will be utilized for immunoaffinity chromatography. The purified protein will then be used in the development of specific immunoassays. We also hope to be able to microinject this protein into hematopoietic cells to determine its effect.

(2) After the SFFV gp52 is purified, we will continue the comparison of the MCF gp70 and the SFFV gp52 in order to gain clues as to why the spleen focus-forming virus is rapidly leukemogenic and the MCF viruses are not. Particular emphasis will be placed on studies to determine if the gp52 is encoded entirely by an MCF-related env gene or whether it is a fusion gene product coded for by a gene in SFFV which originated by recombination between MCF envelope gene sequences and some other unique genetic sequences in the mouse.

(3) Studies will be carried out to determine if antibodies specifically directed to the SFFV-encoded gp52 can protect mice against SFFV-induced disease.

(4). Immunofluorescence assays are being developed to study viral protein expression in individual cells. We hope to be able to determine if viral proteins are expressed in all hematopoietic cells or in only certain cells that are targets of the virus. Immunofluorescence assays will also be useful in detecting a few cells in a culture that are expressing viral proteins, which is not possible using immunoassays on extracts of an entire culture.

(5) Studies on the resistance of various strains of mice to F-MuLV-induced disease will be continued in an attempt to determine the basis for this resistance. Particular emphasis will be placed on the role of MCF-like viruses in this disease.

(B) Studies on FeSV

(1) Studies will be carried out to determine if FOCMA determinants are expressed on any of the gag-x polyproteins encoded by FeSV. If not, anti-FOCMA cat sera will be utilized to detect a second FeSV-encoded protein specifying this antigen.

(2) Using anti-FeSV rat sera, an effort will be made to detect a cross-reacting protein in uninfected cells of a variety of species.

(3) Studies will be carried out to determine the differences in the gag-x polyproteins and the expression of these proteins among transforming and transformation-defective isolates of FeSV.

Publications:

Ruscetti, S., Troxler, D., Linemeyer, D., and Scolnick, E.: Three laboratory strains of spleen focus-forming virus: Comparison of their genomes and translational products. J. Virol. 33: 140-151, 1980.

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Ruscetti, S. K., Donner, L., and Sherr, C. J.: A model for FOCMA expression in cells transformed by feline leukemia and sarcoma viruses. In Fields, B., Jaenisch, R. and C. F. Fox, (Eds.): Animal Virus Genetics, ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Academic Press, Vol. XVIII, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04986-03-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure and Regulation of Mammalian Retroviral and Cellular Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. L. Hager	Head, Viral Immunogenetics Section	LTVG, NCI
OTHER:	E. M. Scolnick	Chief, Tumor Virus Genetics Lab.	LTVG, NCI
	E. H. Chang	Expert	DCB, NCI
	A. I. Oliff	Research Associate	LTVG, NCI
	D. R. Lowy	Medical Officer	DCB, NCI

COOPERATING UNITS (if any)

H. W. Chan	Staff Fellow, RDU, NIAID
M. A. Martin	Head, RDU, NIAID
L. Krueger	Postdoctoral Fellow, Rockefeller University
N. Battula	Visiting Associate, LVC, NCI

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION Viral Immunogenetics Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major emphasis of this project has been the application of recombinant DNA technology to the construction of molecular clones for C-type murine retroviruses, primate retroviruses, and mammalian structural genes. These reagents are to be used for the molecular analysis of mechanisms involved in viral tumorogenesis and eucaryotic gene regulation. Recombinant clones have been isolated and characterized for unintegrated circular intermediates of Harvey Sarcoma Virus (HaSV), Friend Murine Leukemia Virus (FrMuLV), and Baboon Leukemia Virus (BLV), for the integrated copies of AKR Murine Leukemia Virus, and for cellular genes expressed in human reticulocytes. For the C-type retroviruses, the cloned DNA has been shown to be biologically active, either in a transformation assay (HaSV) or in a XC plaque assay (FrMuLV and AKR). Virus isolated from cells transfected with FrMuLV clone DNA produces rapid nonthymic leukemia. Restriction and hybridization analysis of the clones expressed in reticulocytes reveals repetitive sequences interspersed between unique copy sequences.

Project Description

Objectives:

(1) Molecular clones for a variety of B- and C-type retroviruses are to be constructed, both from unintegrated and integrated forms of retroviral-specific DNA.

(2) Structural analysis of specific regions of the cloned viruses is to be performed, particularly regions implicated in the control of virus expression, or in transformation functions.

(3) Biological activity of the cloned sequences is to be analyzed, particularly with regard to the transcription of viral RNA from recombinant genomic or sub-genomic sequences.

(4) An attempt will be made to identify specific structural regions of the retroviral (cloned) DNA sequence that are involved in control of viral expression, such as promoter sites, hormone target sites, etc.

Methods Employed:

(1) The technology for constructing molecular recombinants between the various forms of retroviral DNA and bacterial vectors has been firmly established in the laboratory. Viral replicative intermediate forms of DNA are partially purified by Hirt fractionation and preparative gel electrophoresis. Circular molecules are opened by an appropriate one-cut restriction endonuclease and inserted into a bacteriophage lambda or plasmid vector with the corresponding site. Recombinant molecules are amplified by *in vitro* packaging (lambda) or DNA transformation (plasmid), and recombinant clones are identified by *in situ* hybridization with the appropriate viral cDNA probe. Integrated forms of viral DNA are excised from the cellular genome with a no-cut endonuclease, purified by preparative gel electrophoresis or preparative R-looping, and inserted into the appropriate vector.

(2) Physical mapping of virus recombinant clones is accomplished by digestion of the purified insert fragment with appropriate combinations of restriction endonucleases. DNA regions of particular interest are subcloned to sequence analysis. Further information concerning the physical structure is gained by heteroduplex electronmicrographic analysis between different viral-specific DNA inserts, or by R-loop analysis between DNA fragments and genomic viral RNA.

(3) The biological activity of recombinant molecules to date has been determined exclusively by transfecting the recombinant DNA onto appropriate recipient cells, and scoring the cells for the development of XC plaques in the case of replication-competent viruses, or for foci in the case of transforming

molecules. We intend to extend these methods of detection of biological activity to micro-injection of recombinant DNA directly into Xenopus Oocytes (cytoplasm or nuclei) or into mammalian cells, followed by assays for viral-specific transcription or translation products. We also intend to explore the possibility of transferring fragments of the viral recombinants to mammalian cloning vectors (such as SV40) as an alternative way of looking at the expression of the DNA in mammalian cells.

Major Findings:

Our most important observation to date is that the various forms of viral-specific DNA found either in chronically-infected cells (integrated or endogenous forms) or in acutely-infected cells (replicative intermediates) can be successfully propagated as molecular recombinants with bacteriophage or bacterial plasmid vectors, and that with at least some of the molecules we have constructed, the biological activity of the sequence under consideration is retained. This observation confirms our original proposal that structure-function relationships between specific coding sequences and a corresponding process induced in a recipient cell could be probed with unique precision by the construction of recombinant materials.

In a group of six independently-identified recombinants generated with the HaSV virus circular intermediate, we have identified three unique sizes of molecules. These molecules have been shown to vary only within a certain region of the corresponding virus sequence, specifically the region that has been shown with in vivo material to be redundantly present at each end of the in vivo virus linear molecule. We have demonstrated that the three sizes correspond to molecules containing one, two and three copies of the redundancy tandemly duplicated in the recombinant molecule. The presence of the triple duplication has not been previously observed in any retrovirus system, and suggests that the previously-observed deleted circles might in fact arise by an unequal-crossing-over mechanism. In light of this suggestion, it is particularly interesting that the duplicated and triplicated recombinant molecules apparently undergo extensive crossing-over during replication in the bacterial cell.

Although the structure of the recombinant insert for the Ha-SV clones is permuted with respect to the linear map of Ha-SV RNA, and the non-duplicated clone is missing one copy of the redundancy present at both ends of the virus linear DNA, all of the molecules are infectious in a transfection assay. This raises interesting questions about the mechanism by which the permuted (and/or deleted) molecules could support a successful infectious event, issues that are currently under investigation.

The recombinant clones of the FrMuLV non-defective virus have also been analyzed, both structurally and biologically. Forms corresponding to the non-duplicated and duplicated molecule have been identified. Again, the molecules are found to be biologically active, both in a tissue-culture

transfection assay (XC plaques) and in the animal (leukemogenic).

The recombinant clones for the integrated AKR and MTV sequences have been more recently obtained, and the analysis is only in its preliminary stages. A number of points can be made, however, with data already available. Recombinant copies of the integrated AKR sequences that are infectious in the XC plaque test have maps that are colinear, non-permuted, and essentially identical to the RNA genomic map. Other molecules have been found that contain significant sequence variations, and these are not infectious.

Construction of recombinants for the integrated MTV sequences has been more difficult for technical reasons relating to the unavailability of an appropriate no-cut insertion enzyme. Clones have been constructed from partial Eco RI digests, however, and one recombinant containing a complete copy of the genome has been identified from over 100 independently-derived chimeras. Again, the physical map for this molecule is colinear and non-permuted with respect to the RNA genome, while flanking cellular sequences are unique and apparently random for individual clones. Although this data is consistent with recent data experiments with total cell DNA that have been interpreted in support of the lack of a specific viral integration site, we are looking at the virus-cell joint regions in our cloned material in more detail than is possible with total cell DNA to investigate the possibility of specific virus integration site that would be present in the cell in multiple copies.

Significance to Biomedical Research and the Program of the Institute:

The type of program being developed in this project will provide a new and uniquely powerful approach to the study of the control of RNA tumor virus expression. Techniques and methodologies have deliberately been chosen and developed which would have wide applicability to the study of other virus and endogenous sequences of interest. It is anticipated that the successful attainment of the project goals will have wide ramifications for the study of RNA tumor virus expression in general and for the study of the control of expression of other cellular genetic information as well. The application of recombinant DNA technology to the study of virus genome structure, and especially the study of virus sequence organization in the cellular genome, will lead to a wealth of hitherto unobtainable information on the relationship between viral genetic information and endogenous cellular information and the interaction among these sequences that leads to oncological transformation.

Proposed Course:

The course we propose to follow in our program is to extend the structural and biological analysis of retroviral sequences with a view toward correlation of structural sequence with biological function. Regions on the retroviral genomes (or possibly in adjacent cellular sequences) responsible for specific

functions, particularly functions involved in the control of viral expression will be identified by a variety of approaches: cloning of sub-genomic fragments and assaying for specific function; mutational analysis of genomic sequences to look for loss of function; genetic rearrangement of retroviral "control elements" with other viral or nonviral sequences to generate novel phenotypes; further cloning of integrated viral genomes from cells with altered phenotypes with respect to expression of that viral information. Structural sequences that lead to variant viral expression will then be compared in detail to probe the mechanism by which viral expression is controlled.

Meetings Attended:

Cold Spring Harbor Symposium on the Transformed Cell - 1979 (Invited Speaker)
Gordon Conference on Animal Viruses - 1979 (Invited Speaker)
Cold Spring Harbor Symposium on RNA Tumor Viruses - 1979 (Invited Lecturer)

Publications:

Hager, G. L., Chang, E. H., Chan, H. W., Garon, C. F., Israel, M. A., Martin, M. A., Scolnick, E. M., and Lowy, D. R.: Molecular cloning of the Harvey sarcoma virus closed circular DNA intermediates: Initial structural and biologic characterization. J. Virol. 31: 795-809, 1979.

Lowy, D. R., Rands, E., Chattopadhyay, S. K., and Hager, G. L.: The structure and infectivity of integrated Murine Leukemia Virus DNA. Cold Spring Harbor Symp. Quant. Biol. 44, 1979, in press.

Oliff, A. I., Hager, G. L., Chang, E. H., Scolnick, E. M., Chan, H. W., and Lowy, D. R.: Transfection of molecularly cloned Friend Murine Leukemia Virus DNA yields a highly leukemogenic helper-independent type C virus. J. Virol. 33: 475-486, 1980.

Lowy, D. R., Rands, E., Chattopadhyay, S. K., Garon, C. F., and Hager, G. L.: Molecular cloning of infectious integrated Murine Leukemia Virus DNA from infected mouse cells. Proc. Natl. Acad. Sci. U.S.A. 77: 614-618, 1980.

Chan, H. A., Garon, C. F., Chang, E. H., Lowy, D. R., Hager, G. L., Scolnick, E. M., Repaske, R., and Martin, M. A.: Molecular cloning of the Harvey sarcoma virus circular DNA intermediates: II. Further structural analyses. J. Virol. 33: 845-855, 1980.

Chang, E. H., Maryak, J. M., Wei, C.-M., Shih, T. Y., Shober, R., Cheung, H. L., Ellis, R. W., Hager, G. L., Scolnick, E. M., and Lowy, D. R.: Functional organization of the Harvey murine sarcoma virus genome. J. Virol. in press.

Young, H.A. and Hager, G.L.: Mouse mammary tumor virus and glucocorticoids. In Thompson, E.B. and Lippman, M. (Eds.): Steroid Receptors and the Management of Cancer. Cleveland, Ohio, C.R.C. Press, Inc., 1979, pp. 45-58.

Donehower, L.A., Andre, J., Berard, D.S., Wolford, R.G. and Hager, G.L.: Construction and characterization of molecular clones containing integrated mouse mammary tumor virus sequences. Cold Spring Harbor Symp. Quant. Biol. 44, 1979, (in press).

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Thompson, E.B., Granner, D.K., Gelehrter, T.D. and Hager, G.L.: Unlinked control of multiple glucocorticoid-sensitive processes in spontaneous HTC cell variants. In Cold Spring Harbor Hormones and Cell Culture, Cold Spring Harbor Conferences on Cell Proliferation, 1979, pp. 339-360.

Thompson, E.B., Granner, D.K., Gelehrter, T., Erickson, J. and Hager, G.L.: Unlinked control of multiple glucocorticoid-induced processes in HTC cells. Molecular and Cellular Endocrinology 15: 135, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04987-03-LTVG

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Study of the Molecular Basis of Disease Induced by the Spleen Focus-forming Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Linemeyer	Staff Fellow	LTVG, NCI
OTHER:	E. M. Scolnick	Chief, Tumor Virus Genetics Lab.	LTVG, NCI
	S. K. Ruscetti	Senior Staff Fellow	LTVG, NCI
	A. Oliff	Research Associate	LTVG, NCI

COOPERATING UNITS (if any)
L. Evans and P. Duesberg — University of California, Berkeley, Calif.

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Friend strain of the spleen focus-forming virus (SFFV), a replication-defective murine retrovirus, induces a rapid proliferation of erythroid precursor cells when it is injected as a pseudotype into susceptible mice. Two proteins encoded by SFFV have beeen indentified, a gag gene-related protein and an env gene-related protein, gp52. In order to demonstrate which area(s) of the genome is responsible for the induction of disease, a genetic analysis of this virus has been initiated. The SFFV DNA has been molecularly cloned in a biologically active form. The gp52 protein is expressed in fibroblast cells after transfection and rescue of this cloned DNA. Furthermore, the level of gp52 expression correlates with the latency of disease induced in mice after injection of the virus produced by the transfected cells. Preliminary evidence using a molecularly cloned sub-genomic fragment of SFFV DNA indicates that the sequences necessary for the erythroproliferative disease and those encoding the gp52 are present in the same part of the viral genome; thus supporting the hypothesis that the gp52 is required for the disease.

Project Description

Objectives:

The purpose of this project is to identify the gene(s) responsible for the induction and/or maintenance of the erythroproliferative disease induced by the Friend strain of the spleen focus-forming virus (SFFV), and to examine this gene(s) and its gene product(s) to determine the role it plays in pathogenesis.

Methods Employed:

(1) The entire viral DNA and subgenomic fragments of it have been molecularly cloned in E. coli using the plasmid vector pBR322.

(2) The cloned DNA has been transfected into fibroblast cells and rescued by either superinfection with helper virus or by co-transfection of molecularly cloned and infectious helper viral DNA.

(3) The biolgical activity of the DNA has been monitored by injecting adult NIH Swiss mice with the virus produced after transfection and rescue of the DNA.

(4) The cloned DNA-mediated protection of viral genomic RNA labeled with radioactive phosphorous has been examined by two dimensional oligonucleotide fingerprinting analyses.

(5) The expression of viral encoded proteins has been examined by immune precipitation using various specific antisera.

(6) In vitro mutagenesis of the cloned DNA has been performed by insertion of molecular restriction linker sequences into various sites of the DNA.

Major Findings:

(1) The unintegrated circular form of SFFV DNA has been cloned in a permuted orientation in pBR322 at the unique Hind III site. Clones have been isolated with viral inserts of 6.3kbp containing two copies of the terminal repeat sequences or of 5.7kbp containing only one copy of these sequences. Both types of clones are biologically active, causing the production of erythroproliferative disease-inducing virus after transfection and rescue of fibroblasts with the SFFV DNA released from the plasmid by Hind III digestion. The SFFV biological activity can be rescued from the transfected cells by helper viruses not related to Friend virus, such as Moloney and wild mouse amphotropic type-C viruses, which do not themselves induce an erythroid disease. Virus able to induce the SFFV disease can also be recovered after

transfection and rescue of cloned DNA not released from the plasmid, indicating that the area of the genome which maps at the Hind III cleavage site is not required for the induction of disease.

(2) The SFFV-encoded gp52 is expressed in the transfected fibroblasts as well as in the bone marrow and spleens of mice injected with the virus produced by the transfected fibroblasts. The level of gp52 expression in these fibroblasts correlates to the relative latency of disease induced by the virus which they produce.

(3) A 3.0 kbp subgenomic fragment of SFFV DNA containing the env gene region, the "c" region, and the terminal redundant sequences has also been molecularly cloned. SFFV genomic RNA oligonucleotide protection experiments demonstrate that this fragment contains the SFFV-specific sequences which are not found in the parental Friend helper virus. Preliminary evidence has shown that mice injected with virus produced after transfection and marker rescue of the fragment can induce the same erythroid disease as the original SFFV. Also, further results indicate that the gp52 is encoded within this fragment.

(4) A large number of insertion mutations have been produced in the 5.7 kbp clone of SFFV DNA by in vitro mutagenesis. Molecular clones of these mutated DNAs are being analyzed for biological activity and the sites of mutation are being mapped by restriction enzyme digestion. Results from these experiments should definitively localize the sequences in SFFV required for pathogenesis.

Significance to Biomedical Research and the Program of the Institute:

An understanding of the genetics of leukemia viruses is crucial to understanding the biological activity of these viruses. Molecular clones of various viral DNA species offer the most practical means to study the structure and function of viral genes. This will allow us to determine which viral proteins are responsible for the induced pathogenicity. An analysis of their mechanism of action can then be initiated. Cloned DNA from various viruses can also be used in recombination experiments to study how viruses are formed and to study how different genes interact. Lastly, molecularly cloned subgenomic fragments of DNA can be used to generate specific probes which can in turn be used to detect and isolate related retrovirus genes in other species including man.

Proposed Course:

(1) The genetic analysis of SFFV will be continued by examining the clones of in vitro mutagenized DNA for biological activity after co-transfection with DNA of helper viruses which do not induce erythroid disease in adult mice.

The sites of mutations which affect the biological activity of the SFFV DNA will then be mapped on the genome.

(2) Smaller subgenomic fragments will be molecularly cloned and examined genetically to localize the portion of the viral genome required for induction of disease.

(3) Transfections of hematopoietic cells are being attempted to develop an in vitro assay for SFFV and to investigate whether SFFV alone, without a helper virus, can cause the proliferation of erythroid precursor cells.

(4) Experiments are in progress to map by heteroduplexes the areas of homology and non-homology between the subgenomic fragment of SFFV and a subgenomic fragment of F-MuLV, each of which encode the information required for the disease induced by each respective virus.

(5) DNA probes specific to SFFV will be prepared using cloned DNA fragments to develop assays which could detect homologous sequences in various mouse and non-mouse cells. In particular, hematopoietic cells will be examined to determine the target cells for SFFV.

(6) Using cloned fragments of DNA from different viruses we will attempt to construct new recombinant viruses in vitro and analyze them to study the mechanism of formation of a pathogenic virus.

Publications:

Ruscetti, S., Troxler, D., Linemeyer, D., and Scolnick, E.: Three laboratory strains of spleen focus-forming virus: Comparison of their genomes and translational products. J. Virol. 33: 140-151, 1980.

Troxler, D. H., Ruscetti, S. K., Linemeyer, D. L., and Scolnick, E. M.: Helper-independent and replication-defective erythroblastosis-inducing viruses contained within anemia-inducing Friend virus complex (FV-A). Virology 102: 28-45, 1980.

Linemeyer, D. L., Ruscetti, S. K., Menke, J. G., and Scolnick, E. M.: Characterization of molecularly cloned spleen focus-forming virus DNA. DNA. In Fields, B., Jaenisch, R., and Fox, C. F. (Eds.): Animal Virus Genetics. ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Academic Press, 1980, in press.

Duesberg, P., Evans, L., Nunn, M., Linemeyer, D., Troxler, D., and E. Scolnick: The RNAs of the defective and nondefective components of Friend anemia and polycythemia virus strains identified and compared: Prototypes of a new class of retroviral transforming genes. In Rossi, G. (Ed.): Erythropoiesis and Differentiation in Friend Cells. In press.

Scolnick, E. M., Oliff, A., Linemeyer, D., and S. Ruscetti: Molecular genetics and cell culture assays for helper-independent and replication-defective components of the Friend virus complex. In Fields, B., Jaenisch, R., and Fox, C. F. (Eds.): Animal Virus Genetics. ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Academic Press, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04990-06-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation and Expression of Mammalian RNA Tumor Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. J. Goldberg Microbiologist LTVG, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Endogenous type C virus expression in the rat myogenic cell line, L8, has been characterized. Release of virus was measured during the proliferative and differentiative phase of cultivation. Virus production was unaffected by blocking terminal differentiation but was diminished by inhibitors of DNA syntheses. Exogenous infection of L8 cells with several helper independent type C viruses has been shown to block myogenic differentiation. There was an excellent correlation between inhibition of morphological and biochemical differentiation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

A. To study the expression of endogenous type-C viruses as a function of the growth and differentiation of mammalian cells.

B. To study the effect of exogenous type-C virus infection on the growth and differentiation of mammalian cells.

Methods Employed:

A. Various tissue culture assays and procedures have been employed including: cloning of cells, biological assays for type C viruses, derivation of mutant cell lines, selection of optimal conditions which promote and inhibit cell differentiation, propagation of a variety of RNA tumor viruses in cultured cells, and microinjection of cultured cells.

B. Biochemical methods used include the isolation and purification of viral and cellular nucleic acids, molecular hybridization, synthesis of cDNA transcripts from a variety of RNA tumor viruses, microassays for protein and DNA and the identification and quantitation of mammalian cellular and viral enzymes.

Major Findings:

A. The release of endogenous rat type-C virus has been studied as a function of the growth and differentiation of the rat myogenic cell line, L8. Production of virus was found to be higher during cell proliferation and decreased during terminal differentiation. Non-cytotoxic conditions were described for blocking either DNA synthesis or terminal differentiation. Release of endogenous virus was unaffected by inhibiting cell fusion but was significantly diminished when cells were prevented from entering the 5 phase of the cell cycle. Reversal of the block in DNA synthesis resulted in the resumption of release of maximum levels of endogenous type-C virus.

B. The effect of exogenous infection with helper independent mammalian type-C viruses on myogenic differentiation *in vitro* was characterized. Several viruses were shown to significantly block terminal differentiation. The inhibition was shown to be a function of the level of virus replication. The pattern of differentiation-inhibition was quite different for the different viruses tested. There was an excellent correlation between inhibition of morphological and biochemical differentiation. Myosin heavy and light chain creatine phosphokinase α-actin, and acetyl choline receptor levels were all lower in L8 cultures infected with viruses which blocked myoblast fusion.

Significance to Biomedical Research and the Program of the Institute:

Oncornaviruses have now been isolated from a variety of mammalian and avian species. Many of these viruses have been shown to be involved etiologically in the induction of malignant diseases. Although type C or type B viruses have not been isolated from human cells, subviral footprints have been detected in various human tissues. In addition, type C viruses from several mammalian species have been shown to replicate in and, in some cases, transform human cells. Therefore, it is obvious that these self-contained packages of genes offer a convenient and relevant means to study the mechanism of cell transformation. It is also imperative that the search for a bona fide human tumor virus be pressed. Ultimately this would facilitate a thorough study of the involvement of this class of viruses in human neoplasia and open a rational approach for immunoprophylasix and therapy of these diseases.

Proposed Course:

Studies planned for the next year will concentrate on characterizing the block in differentiation in L8 cells observed following exogenous virus infection. Studies on the expression of endogenous viruses will be expanded to investigate the universality of the basic observation and to sort out the level of virus expression in the various cell types in the culture population. Experiments utilizing the newly acquired technique of microinjection and microsurgery will be initiated.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05064-02-LTVG

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Cloning of Murine RNA Tumor Viruses and Construction of Deletion Mutants in vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. Wei	Expert	LTVG, NCI
OTHER:	G. Hager	Expert	LTVG, NCI
	E. M. Scolnick	Chief, Lab. of Tumor Virus Genetics	LTVG, NCI
	D. Lowy	Sr. Investigator, Med. Ofcr. Dermatology	DB, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Molecular Virology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using recombinant DNA technology, we have constructed a series of insertion-deletion mutants of Ha-MuSV by inserting a SalI linker oligonucleotide into various regions of Ha-MuSV genome with an occasional deletion at that region. By correlating the position of mutation with the transforming ability, we conclude that the sequence responsible for the expression of transforming ability lies within 2.5 kb from the 5' end and that the region between 0.4 kb and 1.5 kb from the 5' end contains essential sequence for transforming functions. The Ha-MuSV sequences can be rescued from the mouse cells transformed by these mutants. We have cloned the Mo-MuLV circular DNA intermediates at SalI site in λgtWES·λB-SalI vector molecules. The restriction enzyme cleavage maps have been determined for these recombinants. Two clones containing full-size Mo-MuLV genome can induce XC plaque forming Mo-MuLV. The Mo-MuLV inserts are infectious only after their excision from the vector DNA. The infectivity of the permuter DNA molecules follows two-hit kinetics, while infection with Mo-MuLV virus follows single-hit kinetics. The potential use of the cloned Mo-MuLV DNA as a vector to transfer genetic markers or to introduce foreign DNAs into mouse chromosomes will be studied.

Project Description

Objectives:

In order to gain insight into the genomic organization and gene expression of murine RNA tumor viruses in mammalian cells, one approach is to use recombinant DNA technology to obtain DNA copies of the RNA tumor viruses. The availability of such cloned DNA molecules enable one to study the viral genes at the nucleotide level and to manipulate the genomes at strategic regions which are involved in tumogenesis and leukemogenesis. Experiments include construction of point and deletion mutants at these regions in vitro. Similar approach could be employed to examine other important viral functions including sequence recognition for integration and gene expression at transcriptional level.

Methods:

(1) Southern's gel blotting technique to identify viral DNAs in a mixture of cellular DNAs.

(2) RPC-5 column chromatography to fractionate DNA fragments according to their AT-GC contents and sizes.

(3) Analytical agarose gel electrophoresis to analyze DNA fragments.

(4) Preparative agarose gel electrophoresis to separate DNA fragments based on molecular size.

(5) Molecular cloning system using lambda phage as vector to obtain viral DNA sequences.

(6) Calcium phosphate dependent transfection assay to assay for transforming and lytic functions of the DNA molecules.

Major Findings:

(1) Using the cloned DNA as a parental genome, we have constructed a series of insertion-deletion mutants by inserting a SalI linker sequence (GGTCGACC) into various regions of Ha-MuSV genome following partial digestion with HaeIII. Following ligation into λgtWES·λB-SalI vector molecules, the mutant Ha-MuSV DNAs were cloned. Fourteen insertion-deletion mutants were restriction enzyme mapped and their biological activities correlated with the locations of mutations. The mutants mapping within 3.0 kb from the 3' end of the Ha-MuSV genome retain full transforming ability. The mutants containing the SalI linker insertion at 0.4 kb or at 1.5 kb from the 5' end also retain transforming

ability, but the number of foci induced by the DNAs in transfection assays is greatly reduced. However, a mutant containing a deletion of 1.5 kb at the 5' end and a mutant with a deletion between 1.0 kb and 1.5 kb from the 5' end completely lost their transforming potential. Furthermore, since Ha-MuSV sequences can be rescued from the mouse cells transformed by these mutants using Moloney murine leukemia virus (Mo-MuLV) as a helper virus, it implies that the in vitro modified DNAs may be converted into genuine mutant viruses.

(2) Four independent Mo-MuLV recombinant clones were isolated. Their DNAs were analyzed by restriction enzyme digestion and gel electrophoresis. Results indicate that three of the clones contain full-size Mo-MuLV genomes of 8.9 kb permuted at the SalI site, including two copies of the terminal repeat sequence (0.65 kb in size). The fourth clone contains 6.7 kb of the 8.9 kb Mo-MuLV genome; a 2.2 kb deletion is located at the 3' end of the genome.

The infectivity of two full-size clones and the deleted clone has been assayed on NIH 3T3 cells using XC plaque test. The deleted clone is not infectious. Both of the full-size clones induce XC plaque forming Mo-MuLV; the Mo-MuLV inserts are infectious only after their excision from the vector DNA by SalI cleavage. The infectivity of the permuted DNA molecules follows two-hit kinetics, while infection with Mo-MuLV virus follows single-hit kinetics as does transfection with non-permuted cloned integrated AKR-MuLV DNA. The results suggest that although the permuted Mo-MuLV DNA contains two copies of the terminal repeat sequence, two Mo-MuLV DNA molecules are needed to register as a single XC plaque.

(3) A 3.5 kb BamHI fragment containing thymidine kinase gene (4K) of herpes simplex virus-1 has been cloned in a plasmid pBR322 and shown to be capable of transforming Ltk^-, a mouse cell line deficient in thymidine kinase, to produce tk^+ clones. In order to study the expression of tk gene under the regulation of Mo-MuLV promotor and to rescue the tk gene as RNA form into RNA tumor viruses, attempts are made to engineer a defective Mo-MuLV virus containing a functional tk gene. The cloned Mo-MuLV DNA was digested with BamHI to remove 3.3 kb of DNA in the middle and the 3.5 kb tk gene was inserted by ligation. The recombinant DNAs were recloned at XhoI site in gtWES B vector molecules. Three Mo-MuLVtk recombinants were isolated. Further experiments are underway to transform Ltk^- with these Mo-MuLVtk DNAs.

Significance to Biomedical Research and the Program of the Institute:

Application of the recombinant DNA technology to RNA tumor viruses would add a new dimension to our understanding of the organization of viral genomes and their biological functions. The methodology developed and biological functions revealed from studies with DNA viruses could be applied to RNA tumor viruses by analyzing their DNA copies from molecular cloning. It is now possible to construct deletion mutants in vitro at defined positions. When these mutants become available, it should be possible to analyze in detail the DNA sequences which are involved in integration, replication and further expression of viral genes.

Proposed Course:

We propose to employ recombinant DNA technology to study the molecular biology of murine RNA tumor viruses. Among these viruses, Ha-MuSV, Mo-MuLV, Ki-MuSV and endogenous viral sequences are particularly interesting to us. Our approach is to isolate these proviruses including linear, circular and integrated forms of viral DNAs from infected cells. These DNA molecules, after being properly enriched and tailored, will be cloned in various lambda-E. coli systems. When these DNA sequences become available we will carry out the following studies: (1) Complete nucleotide sequences of these viruses. These experiments will be performed in collaboration with other laboratories using recently developed DNA sequencing techniques. (2) Construction of mutants. Viral mutant containing specific deletions and point mutations will be constructed from the cloned DNA molecules by modifying these molecules with restriction endonucleases and chemically synthesized linkers. (3) Viral gene expression. Many cloned viral DNA molecules are demonstrated to be biologically active using transfection assays. By examining the deletion location and specific mutations of the mutants constructed, it is possible to define sequences at a nucleotide level that are involved in integration, transcription, RNA processing, tumogenicity and leukemogenicity. (4) Cellular sequences at the viral integration site. By cloning the neighboring genes or DNA sequences adjacent to viral integration sites, we plan to examine the possible regulatory signals and/or structural relationship between cellular and viral genes.

Publications:

Chang, E. H., Maryak, J. M., Wei, C., Shih, T. Y., Shober, R., Cheung, H. L., Ellis, R. W., Hager, G. L., Scolnick, E. M., and Lowy, D. R.: Functional organization of the Harvey murine sarcoma virus genome. J. Virol. in press, 1980.

Wei, C., Lowy, D. R., and Scolnick, E. M.: Mapping of the transforming region of Harvey sarcoma virus genome using insertion-deletion mutants constructed in vitro. In Scott, W. A., and Werner, R. (Eds.): Miami Winter Symposium. 1980, Vol. 12, p. 100.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05066-02-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical Analyses of MuLV mutants and of Fv-1 Restriction of MuLV

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoshio Inoue	Visiting Fellow	LTVG, NCI
OTHERS:	R. H. Bassin	Head, Viral Biochemistry Section	LTVG, NCI
	B. I. Gerwin	Chemist	LTVG, NCI

COOPERATING UNITS (if any)

D. Lowy DB, NCI

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION Viral Biochemistry Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

New cell lines were developed and characterized in order to study the effect of the Fv-1 gene product on transfecting viral DNA. Fv-1 restriction was shown to act at a step prior to formation of normal linear (Form III) viral DNA. Cells which do not synthesize DNA either because of prior treatment with Mitomycin C or following serum starvation remain sensitive to transformation with viral DNA, but fail to support viral replication following infection with MuLV virions. It is likely that transfecting MuLV DNA is able to direct viral replication by a pathway other than that employed by DNA synthesized by infectious MuLV virions.

PHS-6040
(Rev. 10-76)

Project Description

Methods Employed:

Infection with MuLV virions as well as transfection with viral DNA using DMSO or glycerol were both employed in these studies. Cell strains and 3T3-like cell lines were developed from mouse embryo cells as needed. Virus production following both transfection and infection was assayed by a variety of infectious center techniques previously described. Drugs such as Mitomycin C and cycloheximide as well as cell arrest following incubation in serum-depleted medium were also employed to study the effect of cell functions on the viral replicative cycle.

Major Findings:

Transfection was employed to study the effect of Fv-1 restriction on linear MuLV DNA. The standard NIH 3T3 line, usually used for transfection studies because of its relatively high sensitivity, was not considered suitable for these experiments because it is known to possess additional restriction mechanisms. However, two cell lines recently isolated in this laboratory, DBA/2 and BALB/c x DBA/2 possessed the necessary Fv-1 restriction properties and were both highly sensitive to transfection with MuLV DNA. Both the DBA/2 and BALB/c x DBA/2 cell lines were highly susceptible to transfection with NB-tropic MuLV, but the BALB/c x DBA/2 cell line was only 1% as sensitive to transfection with N-tropic AKR DNA as was the DBA/2 cell line. Removal of Fv-1^b restriction in the hybrid cell line by abrogation, however, did not result in an increase in transfecting activity. These results, together with other data from this laboratory, indicate that either (1) Fv-1 restriction operates at a step prior to formation of linear (Form III) viral DNA or (2) that Fv-1 restriction can interfere with viral replication at more than one step in the viral growth cycle.

Cellular metabolic requirements for transfection with MuLV DNA were also studied. Prior treatment of cells with Mitomycin C, an irreversible inhibitor of cellular DNA synthesis, did not block transfection, but, as expected, inhibited infection with virus particles approximately 100-fold. Similarly, cells arrested in G_0 by serum starvation proved to be fully sensitive to transfection with viral DNA even though the same cells are resistant to infection with virus particles. These data, together with other results from this laboratory, show that transfecting DNA is stable in cells which do not synthesize DNA. From parallel experiments we know that infecting viral RNA is not stable under the same conditions. In addition, MuLV infection, even though it proceeds via a DNA intermediate, apparently does not follow the same replicative pathway as does transfecting DNA.

Significance to Biomedical Research and the Program of the Institute:

The transfection studies reported here represent a different approach to the control of virus replication from experiments using virus infection. In the Fv-1 restriction system, it is important to define the point in the viral replication cycle at which the inhibition occurs. The Fv-1 gene represents a highly specific naturally-occurring control system and, if its significance and occurrence in other cells is to be assessed, its exact mechanism of action must be determined.

RNA tumor virus replication is now known to involve a complex series of steps from the entry of viral RNA into the cells, through the formation of various forms of viral DNA, and, finally, to the integration of this DNA into the host cell genome. Little is known at present of the cellular factors involved in this process and the data generated in these experiments should ultimately prove to be of value in the determination of the exact steps necessary for viral replication as well as the role of cellular processes in enhancing or impeding these processes.

Proposed Course:

The Principal Investigator having left NIH upon the termination of his Fellowship, these studies with transfecting DNA are being incorporated into a larger study on the effect of cellular metabolic processes on viral replication.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05067-02-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Regulation of Integration and Gene Expression of Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Das	Visiting Associate	LTVG, NCI
OTHER:	K. I. Wolska	Visiting Fellow	LTVG, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Viral Immunogenetics Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recombinant DNA techniques were employed to construct a defined system to study the control of integration of phage λ: i) pBR322 plasmids carrying the Pint promoter and int gene or Pint promoter and the galK gene were constructed. ii) λCII gene was fused to its pLN operon carried in pBR322 plasmid. iii) The Pint plasmid (Tet^R) and the pLNCII plasmid (Amp^R) were forced to co-exist in E. coli galK$^-$ lysogens with the use of drugs. We find that a) λCII gene product is sufficient to turn on Pint promoter in the absence of cIII gene product in vivo and b) in a coupled transcription-translation system in vitro, CII allows the synthesis of Integrase from Pint-int DNA, or galactokinase from Pint-galK plasmid DNA. The CII protein is being purified and its mode of control on λ integrase synthesis is under study. New plasmid cloning vehicles containing unique SacI, HpaI and BglII restriction sites have been constructed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The key mechanism underlying transformation of cells by viruses is to control the integration of the viral genome and expression of only a part of the genome. E. coli phage λ is a model system to study these regulatory mechanisms. The goal is to analyze the reactions in vitro with purified proteins that regulate these processes and understand their modes of action.

Methods Employed:

Recombinant DNA techniques: Standard procedures included i) restriction of purifed λ or plasmid DNA with restriction endonucleases, ii) removal of the 5'-phosphate moities of DNA fragments with calf intestine alkaline phosphatase, in appropriate cases, iii) joining of DNA fragments with T4-DNA ligase, iv) transformation of Ca^{++}-treated E. coli and selection of transformants, and v) screening of transformants by the restriction analysis of plasmid DNAs.
Assay of λ Integrase: In vivo, integrase was monitored by the measurement of EDTA-and PPi-resistant phages produced by intra-molecular recombination of attP and attB carried in a λ imm^{434} att^{2} phage. In vitro, a plasmid (from K. Mizuuchi) carrying attP and attB was used as substrate and the product of intra-molecular recombination was measured by restriction analysis.
Assay of galactokinase: Formation of ^{14}C-(D)-galactose-1-phosphate was measured using DE81 filters. Extracts were made from heat induced lysogens by lysozyme lysis procedure. Coupled transcription-translation was done in extracts of E. coli made according to Zubay's procedure.

Major Findings:

The following lines of evidence show that the CII gene product of phage λ activates the synthesis of Integrase that is involved in the site specific recombination between phage and bacterial chromosomes. Since the CII protein allows the synthesis of Integrase from the Pint promoter in the absence of CIII gene product, CIII may have a role in controlling the cellular level of CII protein via synthesis or stability:

1. A 0.7 kb BglII fragment of λ carries the functional CII gene. This fragment was inserted into the Amp^{R} Tet^{S} pBR-λpLN- (HindIII-Bam) hybrid plasmid at the BamHI site so that CII gene is expressed when pL transcription is activated by prophage induction.
2. For particular manipulations, the 0.6 kb BalI-PvuII fragment of pBR322 was deleted to give pAD126 Amp^{R} Tet^{R} plasmid that is resistant to both BalI and PvuII. The 5.5 kb λ RI-PvuI fragment containing Pint-int region was inserted into pAD126 to give a Tet^{R} Amp^{S} plasmid.

3. A λ prophage strain (gal1490*∇8∇BamN7N53 CI857∇H1) was doubly transformed by Amp^Rλ pLNCII plasmid and the Tet^R Pint-int plasmid. This strain upon heat induction produced Integrase that allows intra-molecular site specific recombination of attP and attB carried in λatt^2PXB phage.

4. The galK att∇ OP'int^C_{226} region carried in a 5.6 kb SmaI fragment was inserted into the PvuII site of pBR322. This plasmid (pAD105) produces galactokinase from int^C promoter which is CII independent and constitutive. A restriction map of galK region was deduced. Since a 1.7 kb PvuII fragment of pAD105 can be inserted at the PvuII site of pBR322 to allow galactokinase synthesis from Ptet promoter, this fragment must carry a functional galK gene. This PvuII galK fragment was inserted at the single BalI site in the int gene carried in the Tet^R Pint-int plasmid.

5. In vitro in a coupled transcription-translation system, the synthesis of Integrase or galactokinase was induced only when CII containing extract was added. Using this in vitro assay, CII was fractionated.

6. We have constructed new plasmid cloning vehicles containing unique SacI HpaI and BglII restriction sites in the course of the above study.

Significance to Biomedical Research and the Program of the Institute:

Tumor virus induced transformed cells resemble E. coli carrying prophage λ. While the mechanism of transformation of a normal cell to a cancer cell is less understood and under extensive study in our laboratory, an understanding of this simple and well defined procaryotic system will not only facilitate such studies but also give us an idea of how many ways such phenomenon may occur in nature. The N and CII proteins play key roles in regulating the integration and expression of λ genes. Understanding their mechanism of action will be of major significance and application to basic cancer biology especially with the current advancement in recombinant DNA research.

Proposed Course:

Study the interaction of Pint promoter, CII protein and RNA polymerase using genetic and biochemical approaches viz. isolation of $Pint^-$ mutants and RNA polymerase and CII mutants, sequence of the 5' end of int mRNA, CII binding site on Pint promoter region by footprinting experiment.

Publications:

Das, A., Court, D., and Adhya, S.: Pleiotropy of rho mutation in E. coli. In Chakravarty, M. (Ed.): Molecular Basis of Host-Virus Interactions. New Jersey, Science Press, 1979, pp. 459-468.

Gottesman, M., Adhya, S., and Das, A.: Transcription anti-termination by the bacteriophage λ N gene product. J. Molec. Biol., in press.

Das, A., and Wolska, K.: ATP-dependent assembly of oligomeric Rho301 and its dissociation into monomers during ATP hydrolysis. Fed. Proc., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05117-01-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Genetic Analysis of Retroviruses. Control of Replication Transformation and Leukemogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. N. Tsichlis Expert LTVG, NCI

COOPERATING UNITS (if any)

J. M. Coffin Tufts University Cancer Research Center, Boston, Mass.

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Viral Biochemistry Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Distinct biological differences (growth efficiency, transformation) between endogenous and exogenous avian retroviruses were correlated with genomic sequence differences present between these two virus groups. It was found that 1) the viral 3' end (c region) is the single most important determinant of virus growth, and (2) a short sequence close to the 5' end of the viral genome and another sequence between the env and src genes play a control role in the expression of the function of the transforming gene src. We are now involved in work which aims to a more detailed understanding of the functions of these genetic regions and to the examination of their potential involvement in leukemogenesis by nontransforming type-C retroviruses.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The overall objectives of this project are to define the biologically relevant sequences of the retrovirus genome that control viral replication transformation and leukemogenesis.

The specific goals are:

(1) To define biologically important sequence differences between endogenous and exogenous retroviruses and to analyze the role of these sequences in replication transformation and leukemogenesis.

(2) To examine the genetic similarities and differences of the products of well defined endogenous viral loci and investigate the significance of their interactions.

(3) To analyze the significance of the interactions between the defective and the helper component of the acute leukemia-carcinoma viruses in pathogenicity.

(4) To analyze interesting mutant or recombinant viruses obtained in the course of these studies that could further our understanding of the structure and function of the genome of retroviruses.

Major Findings:

(1) Biologically important sequence differences between endogenous and exogenous retroviruses.

Recombinant viruses were generated between RAV-0, one of the endogenous viruses of chickens and PrRSV-B, or td PrRSV-B, or MC-29. We analyzed the recombinant progeny that acquired the env^E of RAV-0 and the transforming potential and/or the rapid growth rate of the exogenous virus. The segregation of nonselected parts of the genome of the parental viruses into the progeny analyzed, has shown that: (a) A short sequence in the gag region and another sequence between the env and src gene of the PrRSV-B genome are associated with transformation. (b) The viral 3' end (c region) is the single most important determinant of viral growth (Tsichlis, P. N. and Coffin, J.M., J.Virol. 33: 238-249, 1980 and Tsichlis, P.N. and Coffin, J.M., Proc. Natl. Acad. Sci. U.S.A., 76: 3001-3005, 1979). On this basis we have redefined the C region as a genetic locus c with two alleles c^x (in the exogenous viruses) that determines rapid growth and c^n (in the endogenous viruses) that determines slow growth rate (Tsichlis, P. N. and Coffin, J.M., Cold Spring Harbor Symposium on Quan. Biol. Vol. XLIV "Viral Oncogenes" 1979, (in press).

In an extension of this work we have recently isolated 20 subgroup E transforming recombinants that lack oligonucleotide 15, the determinant of the PrRSV-B transformation related gag sequence, and 3 recombinants that lack various parts of the sequence in the env-src junction of the PrRSV-B genome. Using well-defined pairs of recombinants that differ genetically only in these selected regions we were able to show that the region mapping between the env and src genes affects the efficiency of transformation (P. N. Tsichlis and J. M. Coffin, in preparation). Preliminary findings related to the gag related sequence indicate that it affects primarily the growth rate of the transforming viruses.

Based on the structure of the integrated provirus, determined by others, and on the role that the C region was shown to play in virus replication, we have postulated that its function is to promote transcription of viral RNA. If that is true, it is conceivable that the c region might be an important determinant of viral leukemogenesis and transformation. This hypothesis is now supported by preliminary evidence.

The role of the C region in the induction of leukemia was suggested by the analysis of the genome of four different RAV-60 recombinant viruses that were found to be highly leukemogenic (Robinson, H. L., et al. Cold Spring Harbor Symposium on Quantitative Biology, Vol. XLIV "Viral Oncogenes" 1979). The evidence presented in detail elsewhere (Tsichlis, P. N. and Coffin, J. M. Cold Spring Harbor Symposium on Quantitative Biology, Vol. XLIV "Viral Oncogenes" 1979 and Robinson, H. L. et al. Cold Srping Harbor Symposium on Quantitative Biology, Vol. XLIV "Viral Oncogenes" 1979) has led us to propose a new testable model for leukemogenesis (Tsichlis, P. N. and Coffin, J. M. Cold Spring Harbor Symposium on Quantitative Biology, Vol. XLIV "Viral Oncogenes", 1979). To strengthen this hypothesis I have tried recently to accumulate evidence that might implicate the viral 3' end in leukemogenesis in other viral systems. The results even though in agreement with the hypothesis are still preliminary.

The role of the c region in transformation was suggested by the following experiments: a mixture of recombinant viruses in which about 50% of the viral genomes had the 3' end of RAV-0 as shown by T_1 oligonucleotide fingerprinting was subcloned in turkey cells in soft agar. Forty-five clones have been isolated and analyzed. So far, I have not yet isolated a transforming recombinant with the 3' end of RAV-0, which suggests the possibility that the c region controls the expression of the src gene. The possibility that recombination cannot take place in this region seems unlikely since I have isolated a number of nontransforming recombinants having the c^x region at the 3' end of the viral RNA in which the cross-over point was probably within this segment of the genome.

In another experiment I have used the same virus mixture and subcloned it on a chemically transformed cell line (QT6 cells) that has a high efficiency of soft agar cloning. Forty nontransforming and transforming recombinants were isolated and are to be analyzed. Identification of a src^+ c^n virus should answer

the imposed question. In addition, if a virus like this cannot transform cells we will have generated important genetic evidence to explain why the endogenous "sarc" is not transforming.

(2) Survey of endogenous viral loci.

We have analyzed the product of seven well-defined endogenous viral loci by T_1 oligonucleotide fingerprinting. The viruses induced from each one of these loci are slightly different from each other suggesting that each locus represents a separate viral entity. Certain genetic regions of the endogenous viruses are very closely related with each other and distinctly different from the corresponding regions of the exogenous virus genomes. One example is the c region.

These differences between endogenous and exogenous viruses identify the endogenous viruses as a separate lineage related but not identical to that of the exogenous viruses. A point of major interest recognized as a result of this study is that in certain lines of chickens the virus expressed is a recombinant between the products of two defective endogenous loci (P. N. Tsichlis, H. Robinson, and J. M. Coffin, in preparation). Our work in the $C_5$7Bl/10 congenic mice has indicated that such viruses generated by recombination between the products of two already existing endogenous virus loci can integrate into the germ line and be transmitted vertically as a new viral genetic locus (Datta, S. K., et al. Immunogenetics 7:359-365, 1978). Such a phenomenon could explain the large number of endogennous virus loci observed in chickens and especially in mice. Interactions between the products of endogenous proviruses are probably of major significance in determining viral leukemogenesis in mice. We plan to take advantage of these phenomena in our studies of virus induced leukemia, as described under the proposed course.

(3) Viral interactions between the defective and the helper component of the acute leukemia-carcinoma viruses.

We have shown that the defective component of MC-29 contrary to the established dogma, can serve as a donor in recombination with the helper virus genome (Tsichlis, P. N. and Coffin, J. M. Proc. Natl. Acad. Sci. U.S.A. 76:3001-3005, 1979). Preliminary evidence suggests that the genome of the defective component of this virus is also altered by recombination with the genome of the helper. Such an interaction could play an important role in leukemogenesis by this class of viruses.

(4) Analysis of new viruses isolated in the course of these experiments.

Two types of variants have been isolated:

(a) Host range variants (Tsichlis, P. N., et al. Proc. Natl. Acad. Sci. U.S.A. 77:536-540, 1980). We have isolated at least three viruses, one of which is a mutant of subgroup B and the others are recombinants between subgroups B, D and E, that have the ability to infect both chicken (C/E) and turkey (T/BD)

cells. All three of these viruses retain the subgroup B or D envelope with a minor alteration that allows it to recognize the subgroup E receptor of the turkey cells. These viruses that seem to be analogous to the polytropic viruses in mice will be of interest in analyzing structure function relationships within the env gene.

(b) Deletion mutants. In the course of this project's experiments we have isolated three deletion mutants of special interest which we plan to further characterize and use as tools in genetic experiments. These are: i) BO10. This mutant has a deletion that involves the env, the pol and part of the gag gene (P27). It retains the src gene the junction sequence between env and src and the 5' end of the gag gene. It makes a small number of particles of an as yet undetermined structure and a large amount of an intracellular gag related protein with a mw of 65000 daltons that does not get processed into the final gag gene products. This indicates that structural alterations of the gag polyprotein could render it insensitive to the action of the protease P15 (P. N. Tsichlis, C. S. Barker, J. M. Coffin, and R. Eisenman, in preparation). ii) BO11. This mutant has a large deletion that involves parts of the gag env and probably all the pol gene. iii) BO12. This deletion mutant lacks the pol gene.

Significance to Biomedical Research and the Program of the Institute:

Our studies have already provided considerable insight into the organization and function of the retrovirus genome. In particular our work has shown for the first time that the expression of certain viral genes (src) is under the control of other viral genetic regions. Outside of the broader implications of these findings, this research could give us an understanding of the pathway that leads into the generation of a transforming virus.

The nonleukemogenicity of RAV-0 combined with the leukemogenicity of other subgroup E viruses that have an exogenous c region indicates the importance of this genetic element in the induction of leukemia in the absence of a transforming gene. In our system we have now generated the tools to test and broaden this observation. If it holds true, a number of models can be tested in order to gain insight into this puzzling phenomenon of leukemia induction by retroviruses. Understanding of viral leukemogenesis could help in understanding leukemogenesis and carcinogenesis in general.

Proposed Course:

(1) Biological significance of the c region.

(a) Establish the role of the c region in leukemogenesis by nontransforming avian and murine retroviruses.

We will use some selected recombinants we have generated, that are identical to RAV-0 but have the exogenous virus c region in order to directly test the

hypothesis that the c region is responsible for the leukemogenic potential of retroviruses. This will be done in collaboration with Dr. H. Robinson. At the same time we plan to extend our observations in the murine system where due to the multiplicity of endogenous virus loci, and the potential for complex interactions between their products, viruses with leukemogenic potential can be generated spontaneously during the lifetime of the animal.

(b) Establish the role of the c region in transformation by transforming retroviruses.

We will continue our efforts, using different methodology if necessary, in order to isolate $src^{+}c^{n}$ recombinants. The isolation of such viruses will directly answer the question of what is the role of the c region in the expression of the src gene.

(c) Dissect the mechanism by which the c region mediates its biological functions. Using the same pairs of viruses that are used in the study of leukemogenesis we will directly test the hypothesis that the c region regulates the transcription of viral RNA. If this hypothesis is correct we will test the model for leukemogenesis we have presented (Tsichlis, P. N. and Coffin, J. M., Cold Spring Harbor Symposium on Quantitative Biology, Vol. XLIV "Viral Oncogenes", 1979.)

(d) Examine the origin of the exogenous virus C region (c^{x}).

(2) Role of other portions of the viral genome in transformation.

(a) The viral sequence between env and src.

Generate viruses carrying a total deletion of this region and study their transforming potential. If impaired, as expected, examine complementation of this function by a variety of nontransforming viruses we have generated that either have a full compliment or a partial deletion of this region.

Examine if an even distantly related sequence is present in the RAV-0 or in other Rous associated virus (RAV) genomes.

Trace the origin of this region in normal cell DNA and examine its expression.

Examine the size of this region using restriction endonuclease mapping of viral DNA.

The understanding of the importance of this sequence that will be accumulated from the experiments proposed here will help us in asking the appropriate questions in order to dissect its function.

(b) The gag gene related control region.

Again we will try to carefully examine the biological properties of transforming viruses that we have isolated that are missing this region of the transforming virus genome. Depending on the results of these experiments we plan to design experiments in order to dissect the function of this genetic element.

(3) Study of the defective acute leukemia carcinoma viruses.

We first plan to establish the alteration of the defective component of MC-29 by recombination with the helper virus. On a later date we plan to use well defined helper viruses with differences in gag and c but identical in the env region and test their ability to alter the leukemogenic potential and/or the disease spectrum of the acute-leukemia-carcinoma viruses.

(4) Analysis of interesting viruses obtained in the course of these studies.

As a first step we plan to continue with the characterization of the B010 deletion mutant. Experiments in progress include electron microscopic examination of the particles released testing of the cleavage of the p65 by p15 and finally examination of the ability of this virus to recombine with non-transforming viruses.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05118-01-LTVG

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structural Analysis of Mouse Mammary Tumor Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. C. Ostrowski	Staff Fellow	LTVG, NCI
OTHER:	L. A. Donehower	Chemist	LTVG, NCI
	G. L. Hager	Head, Viral Immunogenetics Section	LTVG, NCI

COOPERATING UNITS (if any)

G. Schochetman	BCP, FCRC, NCI
J. Lovinger	BCP, FCRC, NCI
C. Garon	LBV, NIAID

LAB/BRANCH
Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION
Viral Immunogenetics Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Molecular clones have been isolated and characterized from integrated copies of mouse mammary tumor virus. Only the 3' half of the genome and portions of the 5' half have been successfully cloned. Evidence from our work and from others indicates a small region in the 5' half cannot be cloned in E. coli. Sequence analysis of the extended terminal redundancy of MMTV indicate that potential regulatory signals exist within the E.T.R. Analysis of the regions of host cell DNA flanking the integrated virus fail to reveal any evidence of potential recognition sites for viral integration.

Project Description

Objectives:

(1) Molecular clones for Mouse Mammary Tumor Virus are to be constructed, both from unintegrated and integrated forms of retroviral-specific DNA.

(2) Structural analysis of specific regions of the cloned viruses is to performed, including restriction mapping and sequence analysis.

(3) Structural regions of the genome are to be correlated with known functions, or functional products, of the virus. Particular attention will be paid to regions of the genome potentially involved in regulation of viral transcription, and to localizing on the genome the sequences responsible for encoding known viral proteins and their translational signals.

Methods Employed:

(1) Molecular recombinants are isolated by inserting viral specific DNA, either from integrated sequences or replicative intermediates, into plasmid or bacteriophage lambda vectors.

(2) Physical mapping of virus inserts is accomplished by restriction endonuclease digestion, followed by gel electrophoresis, Southern transfer, and in situ hybridization with viral-specific cDNA. Regions of particular interest are subjected to DNA sequence analysis by the methods of Maxam and Gilbert, or Sanger (dideoxy).

(3) Known viral proteins are isolated and purified to homogeneity. The amino acid sequences are then determined by standard techniques.

Major Findings:

Molecular recombinants representing the 3' half of the MMTV genome, and portions of the 5' half, have been isolated. Complete sequence analysis of the genome is underway. Initial efforts have focused on the extended terminal redundancy (E.T.R.) present at both ends of unintegrated linear or integrated viral DNA, since it is highly likely that sequences within this region are involved in the promotion and regulation of viral expression. The complete DNA sequence for the E.T.R. (1319 nucleotides) has been determined for three independently derived clones, with essentially perfect agreement for the three clones. Preliminary analysis of the sequence indicates: (1) an open reading frame of 834 nucleotides exists within the E.T.R.; the first initiator codon in this frame would permit the

translation of a protein with 211 amino acids; and (2) at least two sequences with strong homology to the cannonical "Hogness box" (suspected eucaryotic transcription initiation site) are found. Whether these sequences actually correspond to viral mRNA or genomic RNA initiation sites is under investigation.

Additional sequencing of internal regions believed to correspond to the viral proteins gp52 and gp36 is underway. Partial amino acid sequences are available; we therefore will be able to localize the coding regions for the proteins.

Sequence analysis of the regions of host cell DNA immediately flanking integrated viral genomes has failed to reveal any areas of homology that could serve as potential recognition sites for viral integration. Examination of these regions by heteroduplex mapping in the electron microscope, however, has led to the observation of "lollipop" structures indicative of large inverted repeat sequences in every clone so far examined. Whether these structures are simply randomly present in the cell DNA, or are in some way involved in the viral integration process is at present unclear.

Significance to Biomedical Research and the Program of the Institute:

Mouse Mammary Tumor Virus has served as a model system for studying the etiology of a mammary neoplasia induced by an RNA tumor virus. Our long-term goal, the complete structural analysis of the MMTV genome and its RNA and protein products, will provide the molecular framework within which both the oncogenic activity of this virus, as well as the control processes that affect its regulation, can be understood.

Proposed Course:

The complete DNA sequence of MMTV will be determined. Subgenomic probes available from the cloned DNA and a knowledge of the primary sequence will be utilized to investigate the metabolism of viral RNA and protein in infected cells, with particular emphasis on identifying the complete spectrum of proteins produced by the virus (including potential transformation-specific proteins) and on elucidating the mechanisms involved in the regulation of viral expression.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05119-01-LTVG

PERIOD COVERED

October 1, 1980 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Identification and Isolation of the Leukemia Producing Genes in Friend Murine Leukemia Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. I. Oliff	Research Associate	LTVG, NCI
OTHER:	D. Linemeyer	Staff Fellow	LTVG, NCI
	S. Ruscetti	Senior Staff Fellow	LTVG, NCI
	E. M. Scolnick	Chief, Tumor Virus Genetics Lab.	LTVG, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Office of the Director

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Friend murine leukemia virus (F-MuLV) is a C-type retrovirus which produces a rapidly fatal erythroproliferative disease in susceptible strains of mice. We are attempting to identify the precise gene or genes within this virus which are responsible for its pathogenicity. Our approach involves a combination of biochemical, tissue culture, and in vivo animal work. We have isolated and molecularly cloned the entire genome of F-MuLV in a λ bacteriophage vector. This molecularly cloned viral DNA is capable of producing a fully pathogenic F-MuLV upon transfection into mouse embryo fibroblasts. We have molecularly cloned portions of this viral DNA using the bacterial plasmid vector pBR322. One such subgenomic fragment (H-H) consists of approximately 40% of the original parental DNA molecule. This H-H DNA molecule has been transfected into fibroblasts and then rescued by superinfection with a non-pathogenic murine retrovirus (amphotropic 1504-A). This process has yielded viruses which can produce the same disease as seen with F-MuLV. Thus the gene(s) responsible for the pathogenicity of F-MuLV are most likely contained within the H-H DNA fragment we have isolated. Future work is aimed at the isolation of other subgenomic fragments from this H-H piece of DNA in an attempt to identify the smallest possible piece of viral DNA which encodes the disease producing function(s) of F-MuLV.

Project Description

Objectives:

(1) Isolation of the entire F-MuLV genome. Completed.

(2) Molecular cloning of this viral DNA in a form which retains all of the virus' biologic properties. Completed.

(3) Isolation of a subgenomic fragment of the molecularly cloned viral DNA which contains the gene(s) responsible for disease production. Completed.

(4) Further disection of the F-MuLV genome by molecular cloning of smaller subgenomic fragments until the smallest possible fragment is obtained which contains the gene(s) responsible for disease.

(5) Identification of the gene(s) product(s) of this DNA fragment and assessment of the mechanism by which this gene product produces erythroproliferation.

Proposed Course:

(1) Identification of specific restriction endonulease sites within the molecularly cloned fragments of F-MuLV DNA.

(2) Molecular cloning of new subgenomic DNA fragments using these restriction enzyme sites.

(3) Generation of site specific mutations by the insertion of enzyme linker molecules into these restriction enzyme sites.

(4) Transfection of mouse fibroblasts with these subgenomic DNA fragments.

(5) Marker rescue experiments using non-pathogenic retroviruses to retrieve F-MuLV genetic information from the transfected mouse fibroblasts.

(6) Inoculation of mice with newly isolated virus preparations obtained from the marker rescue experiments to assess their pathogenicity.

(7) Identification of the protein products made by these new viral isolates using type specific immunoprecipitation assays, electrophoresis, and autoradiography.

Methods Employed:

(1) Restriction endonuclease mapping of viral DNA.

(2) Agarose gel electrophoresis, Southern blot transfer and hybridization with P^{32} labeled cDNA probes.

(3) Molecular cloning in plasmid vectors.

(4) $CaCl_2$ precipitation of DNA to transfect mouse fibroblasts.

(5) Analysis of virus production from transfected cells by reverse transcriptase and XC-plaque assays.

Major Findings:

(1) The viral DNA of F-MuLV can be cloned as an 8.5 kbp molecule which is both infectious and pathogenic.

(2) A 4.1 kbp subgenomic fragment of this DNA contains the gene(s) responsible for F-MuLV induced disease.

(3) This 4.1 kbp DNA fragment is derived primarily from the 3' end of the viral RNA (genome).

Significance to Biomedical Research and the Program of the Institute:

The localization of the pathogenic functions of F-MuLV to the 3' end of the viral genome is consistent with the data from several investigators which has implicated the 3' end of the retroviral genome in fibroblast transformation in vitro, and in the pathogenicity of MCF viruses. The further use of molecular cloning techniques offers the opportunity to isolate the disease causing gene free from the rest of the F-MuLV genomes, thus allowing the precise identification of the gene product responsible for the erythroproliferative disease observed in mice given F-MuLV. Identification of this gene product hopefully will shed light on the basic mechanism of erythroid cell differentiation, and the control of erythroid stem cell proliferation.

Publications:

Oliff, A. I., Hager, G. L., Chang, E. H., Scolnick, E. M., Chan, H. W., and Lowy, D. R.: Transfection of molecularly cloned Friend murine leukemia virus DNA yields a highly leukemogenic helper-independent type-C virus. J. Virol. 33: 475-486, 1980.

Oliff, A. I., Linemeyer, D., Ruscetti, S., Lowe, R., Lowy, D. R., and Scolnick, E. M.: A subgenomic fragment of molecularly cloned Friend murine leukemia virus (F-MuLV) DNA contains the gene(s) responsible for F-MuLV induced disease. J. Virol. in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05120-01-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Analysis of the Genome Structure and Organization of Avian Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Lautenberger	Expert	LTVG, NCI
OTHER:	T. S. Papas	Head, Carcinogenesis Regulation Section	LTVG, NCI
	P. Tsichlis	Expert	LTVG, NCI
	J. P. Bader	Head, Cellular Transformation Section	LTVG, NCI

COOPERATING UNITS (if any)

J. Rhim — LCMB, NCI
J. Chirikjian — Biochemistry Dept., Georgetown University

LAB/BRANCH: Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION: Carcinogenesis Regulation Section

INSTITUTE AND LOCATION: Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A cDNA probe prepared from an AMV(MAV) RNA template was hybridized to a Southern blot of restriction fragments from cell line Q5 DNA. This line consists of quail cells that have been transformed by avian myelocytomatosis virus MC29 but do not produce virus. The probe hybridized predominantly to a single BamHI fragment and a single EcoRI fragment which suggests that the integrated provirus within Q5 is located at a single site that is stable through cell division. The probe used in this experiment was shown to be virus specific since it predominantly hybridized to restriction fragments in chicken DNA that have been found to contain endogenous virus-related sequences.

Project Description

Objectives:

The scope of this investigation is to determine the role of different portions of the genome of an avian retrovirus in cell type specific transformation. The avian myelocytomatosis virus MC29 has been selected since it can transform fibroblasts in vitro. The techniques of molecular cloning, DNA sequence analysis, and site specific mutagenesis will be used to implicate specific nucleotides in the transformation process. The location of these nucleotides should provide a better understanding of how this virus is able to induce a specific form of leukemia.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells by treatment with proteinase K followed by phenol-chloroform extraction. Preparation of vector DNA from phage λ derivitives by phenol extraction of CsCl banded phage.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose gels.

3. Preparation of cDNA probes using purified RSV or AMV(MAV) RNAs as template and fragmented calf thymus DNA or poly dT as primers.

4. Preparation of nitrocelluose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of the cDNA probe to virus related sequences on these filters.

5. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging.

6. Isolation of phage from the libraries containing virus related sequences by hybridization of cDNA probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure.

Major Findings:

1. DNA from uninfected chickens of the Highline strain contains several loci of virus related sequences. This strain is used by Dr. Joseph Beard to prepare AMV transformed leukemic myeloblasts. The restriction pattern of sequences that hybridize to an AMV(MAV) cDNA probe that occurred in DNA prepared from three embyros from this chicken strain was

quite complex. However, most of the observed bands including all of the predominant bands corresponded to fragments attributed by Astrin to endogenous virus related sequences. The SstI pattern indicates that Highline chickens contain sequences corresponding to the ev1, the ev2, and possibly the ev6 loci of endogenous viral genes.

2. The AMV(MAV) probe used in (1) hybridized to a single BamHI and three EcoRI fragments of Q5 (MC29 transformed quail) DNA. The largest EcoRI fragment (9 kb), however, hybridized most strongly. These results suggest that Q5 contains the viral genome at a single stably-inherited integration site. One of the minor EcoRI fragments may be a junction fragment containing only a small part of the provirus while the other might correspond to sequences in the probe unrelated to MC29. Possible candidates from the later sequences are the ribosomal genes (assuming low level probe contamination) or the cellular genes that are related to the component of the AMV(MAV) complex that causes myeloblastosis.

Significance to Biomedical Research and the Program of the Institute:

MC29 is especially interesting in that it transforms specific target cells in vivo that constitute a very small fraction of the hematopoietic cell population. Since the virus can transform both fibroblasts and bone marrow cells in vitro, it should be possible to determine which viral sequences are required for transformation and whether transformation of bone marrow cells requires sequences in addition to those needed for fibroblast transformation. By comparison of the location of the transforming sequences with the MC29 genetic map, it should be possible to determine the role of the different virus specified proteins in transformation. Understanding the molecular biology of this cell type specific transforming virus should provide general insight into the mechanism of the leukemic process.

Proposed Course:

1. Phage λ derivitives containing the MC29 proviral sequences will be isolated from libraries prepared from partially digested or size fractionated completely digested DNA from MC29-transformed quail cell line Q5. Comprehensive restriction maps will be determined for all such clones.

2. We will determine if mouse 3T3 cells can be transformed by the proviral MC29 sequence within Q5. It is very likely that this will be possible since Copeland and Cooper have been able to transform these cells with DNA from an MC29-transformed mouse nonproducer line. A survey will be made to determine which restriction enzymes do not destroy transformation activity and thus do not cleave within sequences required for transformation. After the recombinant phages have been isolated and the transformation assay has been developed, subgenomic segments from the cloned DNA will be tested for transforming ability. This should define specific sequences required for transformation.

3. Interesting portions of the recombinant phages such as sequences required for transformation will be sequenced. Mutants in these sequences will be isolated by the site specific mutagenesis techniques of Hutchison and Smith or Shortle and Nathans. These mutants will be assayed for transformation ability in order to implicate specific nucleotides in the transformation process.

4. Dr. J. Rhim has isolated RSV transformed human cells. We will determine the structure of the integrated provirus within these cells by hybridization to Southern blots made from restriction digests of DNA from them.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05121-01-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of the Expression of Mouse Mammary Tumor Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. D. Johnson	Guest Worker	LTVG, NCI
OTHER:	M. C. Ostrowski	Staff Fellow	LTVG, NCI
	G. L. Hager	Head, Viral Immunogenetics Section	LTVG, NCI
	L. A. Donehower	Chemist	LTVG, NCI

COOPERATING UNITS (if any)

E. B. Thompson Head, Biochemistry of Gene Expression Section, LB, DCBD, NCI

LAB/BRANCH

Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION

Viral Immunogenetics Section

INSTITUTE AND LOCATION

Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clonal cell lines have been established that contain defined numbers of newly integrated MMTV genomes and have identified phenotypes with respect to the induction of viral RNA expression by glucocorticoid hormones. Analysis of viral RNA metabolism in these cells and in other lines transfected with cloned MMTV DNA is underway. Cloned viral DNA has also been evaluated as a template in an in vitro transcription system, and specific initiation of viral RNA synthesis has been demonstrated. Correlation of viral RNA transcription, both in vivo and in vitro, with potential regulatory information in the DNA nucleotide sequence is the established goal of the program.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

(1) Cell lines containing single copies of MMTV will be isolated, and phenotypes with respect to the level of viral induction by glucocorticoid hormones will be determined.

(2) Molecular clones representing the newly acquired MMTV genomes are to be constructed.

(3) Structural analysis of specific regions of the cloned viruses is to be performed, including restriction mapping and sequence analysis.

(4) Structural regions of the genome are to be correlated with known functions, or functional products, of the virus. Particular attention will be paid to regions of the genome potentially involved in the initiation of viral transcription, and in the regulation of transcription.

Methods Employed:

(1) Cells devoid of endogenous Mouse Mammary Tumor Virus sequences will be infected, clonal lines established, and clones containing single copies of MMTV identified.

(2) Molecular recombinants are isolated by inserting viral specific DNA, either from integrated sequences or replicative intermediates, into plasmid or bacteriophage lambda vectors.

(3) Physical mapping of virus inserts is accomplished by restriction endonuclease digestion, followed by gel electrophoresis, Southern transfer, and in situ hybridization with viral-specific cDNA.

(4) Utilizing the S1 nuclease mapping technique of Berk and Sharpe, probes available from molecular clones of MMTV will be utilized to identify regions of integrated viral DNA that are transcribed into RNA, including potential initiation sites, leader sequences, splicing events. Of particular interest is the identification of initiation sites that are responsive to hormonal regulation.

(5) Using recombinant DNA as a template, attempts will be made to demonstrate specific initiation of viral RNA synthesis in vitro in reconstructed systems. If specific initiation can be shown, efforts will then move to identifying DNA sequences and factors involved in the regulatory processes.

Major Findings

Several rat HTC cell lines have been established that contain limited numbers of newly integrated MMTV genomes,including one single-copy and one double-copy line. The level of inducibility by dexamethesone for MMTV RNA expression has been analyzed, and a wide variety of phenotypes has been observed. Efforts to molecularly clone the MMTV sequences associated with these different phenotypes are underway.

Molecular recombinants containing partial MMTV genomes isolated from the integrated viral copies in chronically infected murine mammary tumor cells have been used to establish transfected lines. NIH 3T3 cells were transfected with a mixture of HaSV cloned DNA and MMTV cloned DNA and scored for foci induced by the HaSV DNA. Lines were screened for potential cotransfection with the MMTV DNA, and several were identified. The site of initiation of MMTV RNA synthesis in one of these lines has been investigated by S1 nuclease mapping, and is found to be in the leftward half of the extended terminal redundancy (E.T.R.). Correlation of this potential initiation site with the DNA sequence, and further analysis of hormone-dependent initiation is underway.

Cloned MMTV DNA has also been utilized as a template for transcription in the in vitro system described by Manley et al (PNAS 76: 160 [79]). Specific initiation has been demonstrated from a cloned DNA fragment containing the rightward E.T.R. The position of RNA initiation again corresponds to a position in the leftward half of the E.T.R. Further localization and characterization of these initiation events is in progress.

Significance to Biomedical Research and the Program of the Institute:

Mouse Mammary Tumor Virus has served as a model system not only for studying the etiology of a mammary neoplasia induced by an RNA tumor virus, but also for investigating the mechanisms underlying hormonal regulation of eucaryotic gene expression. The experimental program undertaken in this project, as well as that in the parallel project (#Z01-CP-05118-01-LTVG) will provide the molecular framework within which both the oncogenic activity of this virus, and the control processes that affect its regulation, can be understood.

Proposed Course:

The complete DNA sequence of MMTV will be determined. Subgenomic probes available from the cloned DNA and a knowledge of the primary sequence will be utilized to investigate the metabolism of viral RNA in infected cells, with particular emphasis on identifying the potential initiation

sites, leader sequences and splicing events. Parallel efforts utilizing in vitro reconstructed systems will be made to further dissect the various structural genomic regions and soluble factors involved in viral transcrtiption and regulation.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05122-01-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Localization and Analysis of the Transforming Sequences of Harvey Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Ellis	Staff Fellow	LTVG, NCI
OTHER:	D. (Defeo) Jones	Microbiologist	LTVG, NCI
	E. M. Scolnick	Chief, Tumor Virus Genetics Lab.	LTVG, NCI

COOPERATING UNITS (if any)

H. A. Young — Biological Carcinogenesis Program
Frederick Cancer Research Center

LAB/BRANCH: Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION: Molecular Virology Section

INSTITUTE AND LOCATION: Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Harvey murine sarcoma virus (Ha-MuSV) is a replication-defective transforming retrovirus composed of both mouse and rat genetic sequences. We have localized the sequences responsible for transformation to ~1.0 kilobases in the 5' half of the viral genome. We have also detected these sequences in the high-molecular weight DNA of both mice and rats. This project has involved recombinant DNA technology and other biochemical analyses.

Project Description

Objectives:

A DNA copy of Ha-MuSV was molecularly cloned in pBR322. A restriction map would be developed for this molecule. Its genetic composition would be compared by hybridization analysis to a DNA molecular clone of rat 30S RNA, which represents the majority of rat genetic sequences in Ha-MuSV and which is nontransforming. The Ha-MuSV rat sequences not homologous to 30S would be deduced as those involved in transformation.

Methods Employed:

1) Transfection of DNA by the calcium precipitation procedure.

2) Analysis of DNA by restriction endonuclease mapping and Southern blot hybridization.

3) Molecular cloning of viral DNA in the plasmid vector pBR322 and in the bacteriophage vector λ.

4) Purification of DNA from phage, plasmid-infected bacteria, and eukaryotic cells.

Major Findings:

1) A detailed restriction endonuclease map was developed for Ha-MuSV and four of its subgenomic fragments.

2) A restriction map was also developed for a molecularly cloned DNA copy of rat 30S RNA and its orientation relative to Ha-MuSV was determined.

3) Hybridization analysis of Ha-MuSV restriction fragments relative to the 30S clone revealed a 0.9-1.0 kilobase pair region in the 5' half of the Ha-MuSV genome nonhomologous to 30S.

4) These nonhomologous sequences were shown to be essential for both cell transformation as well as encoding of the transformation-specific protein p21.

5) These nonhomologous sequences are highly conserved, being present in cellular DNA in only one to several copies in a wide variety of species, including rat. In contrast, the 30S sequences are present in multiple copies in rat and mouse and in fewer copies in other species.

Significance to Biomedical Research and the Program of the Institute:

Ha-MuSV is rare among transforming retroviruses in that most of its non-helper sequences are not required for transformation. The role of these nontransforming (30S) sequences in the biology of Ha-MuSV is not known, although they must play some vital role in that they were positively selected during the generation of Ha-MuSV. Insofar as these sequences flank the region required for transformation, they provide a useful model system for studying the influence of well-characterized flanking sequences upon viral transformation.

Proposed Course:

1) Comparison of the transforming region of Ha-MuSV to those of Kirsten-MuSV and rat sarcoma virus.

2) Cloning of the endogenous rat gene homologous to the transforming sequences of Ha-MuSV and characterization of its flanking sequences.

3) Sequence analysis of the viral and endogenous rat transforming genes.

Publications:

Chang, E. H., Maryak, J. M., Wei, C.-M., Shih, T. Y., Shober, R., Cheung, H. L., Ellis, R. W., Hager, G. L., Scolnick, E. M., and Lowy, D. R.: Functional organization of the Harvey murine sarcoma virus genome. J. Virol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05123-01-LTVG

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Control of Transcription Termination

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. I. Wolska	Visiting Fellow	LTVG, NCI
OTHER:	A. Das	Visiting Associate	LTVG, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Tumor Virus Genetics

SECTION Viral Immunogenetics Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Biochemical and genetic analyses of Rho protein of E. coli revealed the following mechanism of transcription termination mediated by Rho: 1. The dissociation of ribosomes at the nonsense codon located at the end of a cistron triggers Rho-mediated transcription termination. 2. Hexameric Rho binds to the nascent RNA·RNA polymerase·DNA complex at the termination site and interacts with the RNA polymerase to displace it from the 3'-end of the RNA. 3. The resulting hexameric Rho·RNA complex, as a result of ATP hydrolysis, dissociates into free RNA and monomeric Rho. 4. Monomers of Rho undergo ATP-dependent assembly and the newly formed hexameric Rho then participates in a second termination event. This model of Rho-mediated termination, supported by the experiments described here, outlines catalytic action of Rho for the first time.

Project Description

Objectives:

Transformation of cells are initiated by a change in the pattern of gene expession. Termination of transcription plays a major role in the control of gene expression. The purpose of this study is to understand the basic cellular mechanisms of control of gene expression in a well defined system such as in E. coli and its phage λ .

Methods Employed:

Genetic Techniques: Isolation and characterization of conditionally lethal mutants of E. coli defective in Rho protein using standard phage and bacterial genetic techniques.
Biochemical Techniques: i) Purification of Rho protein from E. coli by standard enzymological techniques; ii) assay of RNA-dependent ATPase activity of Rho by measuring ^{32}P-phosphate produced by the hydrolysis of γ-^{32}P-ATP; iii) in vitro transcription of λ DNA; iv) kinetics of heat inactivation of Rho; and v) determination of the subunit structure of Rho by sephadex gel filtration.

Major Findings:

1. We have isolated a number of E. coli temperature sensitive mutants defective in transcription termination in vivo as a result of lesion in the structural gene for the Rho protein. Rho isolated from these mutants were found to be i) defective in transcription termination on λ DNA template, ii) temperature sensitive for its poly(C)-dependent ATPase activity, and iii) defective in oligomerization for the formation of hexameric Rho. These mutants are defective in displaying the polarity in gene expression of several polar mutations in a number of operons. These results proved that translation termination is a prerequisite for transcription termination. Our previous studies with RNA polymerase mutants established a functional interaction between Rho and RNA polymerase in transcription termination.

2. In order to examine whether the inability of a mutant Rho protein to form hexamers is the basis for its being temperature sensitive, we have i) analyzed the oligomerization of Rho and ii) studied the rate of heat inactivation of Rho isolated from the wild-type and mutant cells. In brief, we find that Rho monomers are heat labile compared to oligomers and that heat lability can be a measure of the oligomeric state of Rho. This conclusion was based on the following results. a) Wild type Rho, which is normally a hexamer, formed monomers upon dilution. Monomeric Rho^+ was heat labile compared to hexameric Rho and monomeric Rho^+ could be protected from heat inactivation in

the presence of ATP. b) Rho15 and Rho301 mutant proteins did not normally form hexamers. Upon treatment with ATP, these two proteins formed tetra- and hexamers. These two proteins were also protected from heat inactivation in the presence of ATP.

3. Studies with Rho301 mutant protein strongly suggest that hexameric Rho•RNA complex dissociates into monomeric Rho and free RNA as a result of ATP hydrolysis and that ATP hydrolysis catalyzed by Rho involves a cyclic process of dissociation and reassembly. These studies also suggest that Rho301 is defective in this cyclic process. The above conclusions are based on the fact that i) RNA-dependent ATP hydrolysis by Rho301 led to the increased heat lability of this protein, and ii) Rho301 required at least 10 times more RNA to catalyze unit amount of ATP hydrolysis compared to Rho^+ or Rho15. The second observation can be explained if Rho301 is defective in its interaction with RNA or its oligomerization is a rate limiting step in RNA-dependent ATP hydrolysis catalyzed by this protein. These two possibilities, clearly exclusive to each other, are under study.

Proposed Course:

Further biochemical studies to understand the mechanism of Rho action outlined above.

Significance to Biomedical Research and the Program of the Institute:

Our studies are intended to unravel a key mechanism of gene expression that is central in the Institute's effort to understand the basic biology of cancer cell. Understanding a basic cellular process such as transcription termination has a direct bearing on our Institute's outstanding goals.

Publications:

Das, A., and Wolska, K.: ATP-dependent assembly of oligomeric Rho301 and its dissociation into monomers during ATP hydrolysis. Fed. Proc., in press.

SUMMARY REPORT

LABORATORY OF VIRAL CARCINOGENESIS

October 1, 1979 to September 30, 1980

The Laboratory of Viral Carcinogenesis (1) plans and conducts research on carcinogenesis with emphasis on virus-host relationships and on the detection and characterization of oncogenic viruses and the mode of viral transmission in animals and in man; (2) studies the nature, location, and mechanisms of action of viral and cellular genes related to genotypic and phenotypic expressions of malignancy in cells; (3) studies the host immune mechanisms related to the control of cancer; and (4) conducts investigations on the molecular processes of viral carcinogenesis and co-carcinogenesis. Intramural and collaborative research efforts employing interdisciplinary approaches have produced improved understanding concerning the nature of cellular transformation. Several significant laboratory results have brought certain types of human cancer within range of more meaningful laboratory and clinical etiological research. Recent clinically oriented results obtained by this laboratory are summarized first, followed by those with clear implications for human cancer and last, those exploring the basic etiological and host mediated mechanisms of malignant transformation.

Clinically Oriented Findings:

Skin testing for melanoma and breast cancer: It was demonstrated in 40 cancer patients that crude membrane extracts from 3 different human melanoma lines, infected with vesicular stomatitis virus and UV inactivated, gave markedly more sensitive and specific cancer skin test reactions than membranes from non-virus-infected cells. Positive skin tests occurred in 17 of 29 (85%), 11 of 20 (55%), and 13 of 18 (72%) tests respectively, performed in 20 melanoma patients. The same cell membrane extracts that had not been infected with virus gave positive skin tests in 2 of 20 (10%), 4 of 20 (20%), and 2 of 18 (11%) tests, respectively. The three virus-augmented extracts were positive in less than 6% of the tests in control subjects with other cancers. The most promising of clinical studies conducted after this initial positive series has been with virus-augmented cell membrane extracts of cultured breast cancer cells. Whereas 14 of 33 (42%) breast cancer patients gave positive skin tests with material prepared from 5 different breast cancer lines, only 3 of 33 (10%) patients with another tissue type of cancer (lung or melanoma) gave positive tests. One breast cancer cell line (MCF-7), gave positive skin tests in 7 of 7 breast cancer patients, and only 1 of 7 patients with other cancers.

Burkitt's Lymphoma: The American Burkitt Lymphoma Registry operated by this Laboratory collected an additional 150 cases and summarized the results on 256 American patients with this lymphoma. Disease presentation was related to the age of the patient and rapidly growing tissues provide the major site of action. Further evidence was found for the existence of different variants of this disease having a close association with Epstein-Barr virus (EBV) as suggested by high EBV serum titers. These patients have a better prognosis

than those not showing evidence for the presence of EBV. A genetic predisposition was suggested by an increased familial incidence of cancer in first degree relatives and the occurrence of Burkitt's lymphoma in families with X-linked recessive lymphoproliferative syndrome. A high rate of curability and a low incidence of late relapse were observed in the American cases vs the African cases. This suggested that reinduction probably occurs frequently in the African patients because of prominent continuous environmental etiological factors. This is supported by the shifting pattern of disease presentation in Ghana. As abdominal tumors increase jaw tumors decrease and the disease appears in older groups with an overall decline in incidence. These changes appear attributable to improved malarial chemoprophylaxis. The first documented EBV-induced lymphoma in a non-human primate (white-lipped marmoset) was accomplished providing a clear model for study.

Nasopharyngeal Carcinoma (NPC): Studies utilizing the American NPC Registry led to the improved diagnosis and management of NPC patients in the USA where approximately 2000 new patients appear yearly. Multidisciplinary studies of this disease have been expanded to include the Mayo Clinic, Walter Reed Army Medical Center, and the Bethesda Naval Medical Center. IgA antibody titer to EBV was shown to have clinical value and EBV was shown to be more closely associated with undifferentiated NPC than with squamous cell NPC.

Breast Cancer: A collaborative effort between this Laboratory and the Institute Salah Azaiz in Tunisia was continued for the study of the pathogenesis and treatment of rapid progressing breast carcinoma (RPBC). This disease, characterized by edema and inflammatory signs, has provided a study model for aggressive breast cancer. This form of breast cancer has proven to be unresponsive to conventional therapy (surgery and radiation). Combination chemotherapy consisting of 5-fluorouracil, methotrexate, and cyclophosphamide has resulted in major tumor regression in over 75% of the patients. High levels of progesterone receptors were associated with favorable response to treatment. Immunological surveys showed that Tunisians had a higher reactivity to a wide variety of antigens than Americans. Tunisian RPBC patients had a vigorous immune response to the tumor- and murine mammary tumor virus-associated antigens while American breast cancer patients tended to have suppressed immunity, especially those with advanced disease.

Findings Closely Applicable to Human Cancer:

This laboratory recently presented evidence that a line of human fibrosarcoma cells (8387) produces a growth factor related to multiplication stimulating activity (MSA), a growth factor first described as a product of a rat liver cell line grown in serum-free medium. MSA is a member of the insulin-like growth factor family, the somatomedins, and shares with these polypeptides the ability to interact with the same receptor in various tissues and to stimulate growth-related processes of certain cells in culture. The isolation and purification of 8387-MSA has now made possible the elucidation of the primary structure in order to compare its structural features with those of the somatomedins and to prove or disprove whether tumor produced growth factors are different from normal peptide hormones. The development of radioimmunoassays will allow us to screen tumor tissues for MSA-like material.

New strategies and methodology have been developed or adapted for the detection, isolation, and characterization of oncogenic viruses associated with primates. This includes virus-related genetic sequences detectable in whole or in part in the nucleic acids of normal or malignant cells derived from primates, including the human. There are certain primate species from which a particular class of endogenous retrovirus is isolated with high frequency (baboons among the Old World primates and squirrel monkeys among the New World primates). There are other groups of animals from which endogenous retroviruses have been obtained by similar methods, but only at low frequency. The probability of virus isolation, therefore, does not appear to be related to the number of endogenous DNA copies detected or to the levels of viral-specific RNA expressed. Both the low probability of isolation and the very long period needed to detect virus replication suggest that still other primate species will yield endogenous viruses. These may be genetic relatives of the viruses previously isolated or may represent members of some as yet undetected retroviral classes. It also appears that a majority of primate species are capable only of low frequency virus activation.

Three new viruses, isolated from primates closely related to man (langur, owl and stumptail monkeys) were biochemically and immunologically characterized. They are endogenous, genetically transmitted viruses; thus the viral information was detectable in the DNA of normal cells. It was demonstrated that related species have related viral genes in their DNA and that those gene sequences have evolved with the species and are contained in the DNA of both New World and Old World primates. These isolates have brought the total of genetically transmitted primate viruses to 5 (3 type C and 2 type D). None of the new type C viruses are closely related by hybridization or by immunologic parameters to the previously described baboon type C viruses or to the squirrel monkey endogenous type D viruses. A single Old World monkey species, for example, the African green monkey, can be shown to contain multiple copies of at least three distinctly different retroviral classes (two type C and one type D) in its normal cellular DNA. Cell lines in culture that are not releasing virus can be shown, nevertheless, to be transcribing viral-specific RNA. The fact that viruses have been isolated from man's closest relatives and that related DNA sequences have been detected in human DNA, suggests that as technology improves and experience is gained, other primates, including man, will most likely yield these types of endogenous oncogenic viruses. The existing isolates have made possible the development of biochemical and immunological assays for markers related to viral gene expression in a variety of human normal and malignant tissues.

Two new type C viruses were isolated from mice originating from Southeast Asia. The unusual finding concerning these isolates was their demonstrated preferential relationship to primate type C viruses (woolly monkey and gibbon ape) rather than to the classical murine type C oncogenic viruses. The fact that endogenous viruses are genetically transmitted in animal species has served to demonstrate the evolutionary progression of oncogenes in the various species, including primates and man, and how interspecies transmission and recombination of oncogenic viruses may occur.

The methodology for the in vitro synthesis of human monoclonal antibodies from human-mouse hybridoma cell lines was developed. These human hybridomas were obtained by the fusion of human lymphocytes (from sections of lymph nodes from mastectomy patients) with nonproducer murine myeloma cells. The antibodies produced were either human IgG or human IgM at levels between 0.1 and 20 micrograms of immunoglobulin per ml of culture fluid with the duration of synthesis ranging to 130 days post fusion. Some of the immunoglobulins were shown to react by the immunoperoxidase technique with human mammary tumors (including the tumor from the patient from which the segments of nodes were obtained). These same antibodies did not react with normal mammary epithelium found in the same breast. The specificity of this reactivity is under further investigation.

Three different human tumor lines in culture, a rhabdomyosarcoma, a bronchogenic carcinoma and a metastatic melanoma, release proteins (transforming growth factors, TGFs) into the medium that confer the transformed phenotype on untransformed fibroblasts. These proteins are acid and heat stable, produce profound morphologic changes in rat and human fibroblasts and enable normal anchorage-dependent cells to grow in agar. Each of the TGF activities is destroyed by trypsin or dithiothreitol but is stable at 100°C for 2 min and to repeated lyophilization from 1 M acetic acid. Removal of the transforming protein results in a reversion of cell phenotype. The major activity interacts with epidermal growth factor (EGF) cell membrane receptors. The peptides from these tumor cells are similar in their action to sarcoma growth factor (SGF) released by murine sarcoma virus-transformed rodent cells. The most anchorage-independent tumor cells also released the most TGFs. Production of these factors is not restricted to RNA tumor virus-transformed cells, sarcoma cells or rodent cells and may be a more general expression of the transformed phenotype. In assays comparing growth stimulation of mouse, rat, and human fibroblasts in monolayer cultures there is no evidence for species specificity of the factors produced by human cells. TGF production by transformed cells and the responses of their normal counterparts raise the possibility that cells "auto-stimulate" their growth by releasing factors that rebind at the cell surface. Experiments demonstrating that growth in soft agar of tumor cells depends on the number of cells seeded per unit area argue that diffusible substances released by cells stimulate neighboring cells. Those cells that grow best in soft agar are the most efficient producers of transforming peptides. The approach described here offers a sensitive assay for growth-stimulatory factors associated with maintaining the transformed state. Purification of such factors may lead to the development of specific immunologic assays for their production by tumor cells and their presence in body fluids. The factors may be analogous to peptide growth factors expressed early in normal embryonic development. These factors, like SGFs, are EGF-related peptides as insulin and somatomedins are related and appear to have evolved from common ancestral proteins. Further purification of these growth factors from human tumor cells is needed to define their relationship to other biologically active peptides that cells produce.

A system was developed to study the rescue of transforming genes from cells in culture. Preliminary studies with primate tumor cell lines suggests that it may also be applicable to the isolation of tumor genes from human cells.

A library of such cancer genes isolated as part of the genome of defective type-C viruses should be useful for our understanding of mechanisms of malignant transformation and may at the same time provide material for the development of immunotherapeutic reagents. An immunotherapeutic approach to cancer prevention, reflected in our current work, appears feasible since (1) it has been demonstrated in several animal systems that products of virus coded transforming genes can act as tumor-specific transplantation antigens and (2) it is now possible to generate stable cell lines, (hybridoma lines), which continuously produce specific high titered, monoclonal antibody in-vitro. Since type-C viruses are part of the genetic makeup of virtually all vertebrate species including primates and overt expression of these genes, such that infectious virus is produced at high titer, they are generally associated with an increase in the frequency of tumor formation. Natural tumors, commonly harbor variants of the endogenous type-C virus which have acquired a transforming gene. Thus, the endogenous type-C virus has the capacity to act as a transductant of at least some cellular genes, those which can endow the virus with the capacity to transform cells to malignancy. The frequency with which such transforming genes are acquired by type-C viruses appears to increase when infected animals are treated with chemical carcinogens.

Basic Oncology Findings:

A hormone-like cell growth factor (Sarcoma Growth Factor - SGF) was detected, isolated, and characterized. SGF effected a reversible malignant phenotypic change in cultured cells. These studies were done to determine how these substances biochemically bring about the morphological phenotype of transformed cells. Serum-free conditioned media was searched for growth factors from MSV-transformed 3T3 cells. An acid soluble peptide extract stimulated ^{3}H-thymidine incorporation into the DNA of serum depleted cells, competed with radiolabeled EGF for plasma membrane receptors, and stimulated anchorage independent growth of untransformed cells in soft agar. Comparison of SGF and Epidermal Growth Factor (EGF) showed that while they share some common properties they are distinctly different. Both interact with the EGF membrane receptor system, are heat stable peptides requiring disulfide bonds for activity, and are active in nanogram quantities. They differ in that SGF allows soft-agar growth where EGF will not; SGF is produced by MSV-transformed cells whereas EGF is not. Antibodies directed against EGF do not recognize SGF, and their isoelectric points are distinctly different, EGF being 4.4 and SGF being 6.8.

Mammary tumor virus (MTV) host range variants were selected and characterized. They have the ability to efficiently infect cells and actively produce viral progeny. This is a major breakthrough in MTV virology and the study of breast cancer because it has permitted the development of cell substrates for mammary cell infectivity and in combination with chemical carcinogens and tumor promoters, the development of in vitro transformation assays for mammary epithelial cells.

Extrachromosomal DNA purified from mink cells acutely infected with ST-FeSV was electrophoretically separated, transferred to a solid substrate, and hybridized with radiolabeled DNA transcripts complementary to different portions

of the FeSV and FeLV RNA genomes. DNA species of 8.5 and 5.0 kilobase pairs (kbp) in length were shown to represent the linear, unintegrated intermediates of ST-FeLV and FeSV, respectively.

Restriction endonuclease sites for seven enzymes were mapped within FeSV DNA and three other enzymes were identified which fail to cleave the provirus. Using DNA transcripts complementary to different portions of the FeSV genome, FeSV src sequences were positioned within 2.0-3.4 kbp from the 5' end of proviral DNA (with respect to the 5'-3' orientation of the RNA genome) and were found to be flanked by FeLV-derived sequences. Like other type C proviruses, FeSV and FeLV DNA intermediates are longer than viral RNA and contain sequences at each terminus derived from both the extreme 3' and 5' ends of viral RNA. The order of sequences can be designated 3'5'____3'5' where 3'5' signify the 0.7 kbp extended, terminally redundant (ETR) sequences and "_____" represents viral structural genes.

The FeLV-derived sequences at the 5' end of FeSV were found to originate from sequences present at the extreme 5' end of FeLV; by contrast, FeLV-derived sequences distal to the 3' end of the src gene are derived from two short, discontiguous segments of the FeLV genome. In collaboration with Dr. George Vande Woude of the Laboratory of Molecular Biology, NCI, FeSV and FeLV DNA intermediates were cloned in a prokaryotic vector and propagated in a bacterial host. DNA fractions containing 8.5 and 5.0 kbp viral DNA were cleaved in the ETR regions with Eco RI and ligated to lambda "arms" prepared from the EK-2 certified vector, ggtWES"gB. The ligated DNA was packaged in vitro into plaque-forming phage particles, and recombinant phages containing FeSV and FeLV DNA "inserts" were isolated by "plaque-blot" techniques. The hybrid phages (designated gST-FeSV and gST-FeLV) were characterized by restriction enzyme analyses, R-looping with purified, polyadenylated viral RNA, and by heteroduplex formation. As expected, the recombinant phages contain all of the genetic information represented in FeSV and FeLV RNA genomes, are 0.7 kbp shorter than their respective proviral DNA intermediates, and contain one permuted ETR sequence. In agreement with nucleic acid hybridization studies performed with FeSV restriction fragments, src sequences were localized within 1.9-3.4 kbp from the 5' end of the DNA provirus.

12-O-tetradecanoylphorbol-13-acetate (TPA) and certain analogues may have some structural resemblance to an endogenous growth promoting and differentiation modulation substance(s) that have specific membrane receptors. TPA recognizes and interacts with these receptors, thus mimicking the action of the putative substance(s). Furthermore, TPA may induce and regulate the production of the endogenous substance(s). TPA can also increase cell susceptibility to a normal regulator of cell growth and modulator of cell differentiation. Several biochemical and biological studies provide evidence that the initial site of action of tumor-promoting phorbol esters may be the membrane of target cells. The tumor-promoting phorbol esters have been found to modulate the interaction between epidermal growth factor (EGF) and its membrane receptors in a variety of cells in culture. The pleiotypic effects of TPA and the related tumor promoters in-vivo as well as in-vitro mimic several actions of growth stimulating polypeptide hormones such as EGF and SGF.

A fibroblastic (MMC-F) and an epithelial (MMC-E) cell lines were established from a single embryo of the wild mouse strain Mus musculus castaneous. Clonal derivatives of these lines have been grown for many serial passages without any significant morphological changes. These cell lines do not express antigens related to known type-C viruses and are resistant to activation by bromodeoxyuridine. We have classified the MMC-E cell line (clone 7) as epithelial. Both cell lines are susceptible to chemical as well as type-C viral transformation in-vitro. Inoculation of transformed MMC-F cells into nude mice gave rise to fibrosarcomas, whereas transformed MMC-E cells produced poorly differentiated carcinomas. The availability of a set of stable, non-transformed syngeneic mouse cell lines which differ in their phenotype should prove useful for studies on the mechanisms of malignant transformation. The epithelial cell line, MMC-E, in particular, should be helpful in the study of chemical as well as viral carcinogenesis in view of the fact that the majority of human tumors are of epithelial origin, and an appropriate epithelial cell culture system has thus far been unavailable.

Colonies of Asian species of Mus established in this Laboratory were used as a basis for the study of genetic variability of murine endogenous retroviruses and the genetic elements which affect their expression and/or involvement in neoplastic disease. Four different classes of endogenous retroviruses have been isolated from Asian Mus species. These include two distinct classes of type-C virus (designated C-I and C-II), a novel class of viruses (M432 group) and milkborne type-B viruses. The type-C and M432 group of viruses were obtained either from cultured cell lines which spontaneously began producing virions or by induction of tissue culture cells with pyrimidine analogues followed by cocultivation with sensitive indicator cell lines. The major internal viral protein (p30) of the C-I class competes in a woolly monkey - gibbon ape group-specific radioimmunoassay, whereas, the respective protein of the C-II class shows no competition.

Examination of the composition and products of individual virus genes of several prototype murine recombinant viruses showed that postulates of causality could be met. Some recombinant viruses could induce in pure form lymphomas from which only the input virus could be isolated, and such purified virus in turn could induce the same disease. The viral envelope gene product (gp70) was found to be different from known ecotropic and xenotropic viruses by a number of biological and biochemical criteria, such as neutralization, interference and oligopeptide mapping. Some of these recombinant viruses were found to have either a part or the whole envelope gene exchanged or recombined. A series of new polytropic recombinant viruses were isolated from various Moloney leukemia virus stocks as well as from Gross and Graffi leukemia viruses and interestingly enough from C57 Black mouse cells uninfected with exogenous leukemia viruses. Each subset had unique properties and some of these RM's did not have classic xenotropic peptide spots. Additionally, several of these evidently have further sites of recombination in their gag regions. This family of RM viruses was not homogenous in respect to host range properties. Some isolates were completely polytropic and some were almost ecotropic. The recombinant virus genome was found coated with ecotropic envelopes and therefore insusceptible to type specific antibody and natural protective factors but it could, with its own envelope overcome the ecotropic virus interference

barriers. Because some apparently pure ecotropic virus stocks have large amounts of masked RM virus, purification of antigens and interpretation of immune responses must be evaluated with caution. It was clear that masking protected the RM virus from the oncornavirus inactivating factor (OIF) found in normal mouse sera. This phenomenon of masking has now been observed in every MuLV leukemogenic stock tested despite a variability in growth avidity of the RM component. This general phenomenon also has ramifications to virus purification procedures where selection pressure against one virus causes the adoption of the coat of the favored virus.

Examination of the natural defense mechanisms in the mouse which are directed against xenotropic and recombinant viruses showed that potent, non-immunoglobulin mediated inactivation of xenotropic and RM viruses could occur on treatment with pure lipids found in the very light density lipoprotein fractions of mouse sera. All mouse sera, but not other mammalian sera, had oncornavirus inactivating factor (OIF), including those which were previously reported to have been negative. In the latter, OIF could be shown to be complexed into an inactive form in serum. Once purifed, OIF had a broader spectrum of activity which included some ecotropic viruses as well as some leukemia viruses of other species. No reduction of OIF could be demonstrated in preleukemic states in irradiation induced lymphomas of mice, or naturally arising AKR mouse lymphomas. However, it appears reasonable that OIF is the basic reason for genomic masking of recombinant murine leukemogenic viruses.

A major finding has been in the area of virus negative murine leukemia. Irradiation of mice of various strains, including virus negative NIH Swiss, resulted in a high incidence of thymic leukemias which appear not to express virus or viral antigens. Cultures of NIH Swiss mouse X-irradiation induced thymoma (NIXT) cells were free of virus or known viral proteins. Because of this cloned cell line's insusceptibility to entry and replication of all RM viruses, but not to ecotropic viruses, it was found that these cells contained on their surface an easily removable glycoprotein (Ngp70) which was related primarily to RM gp's by immunological and peptide mapping techniques. This gp70 found in all subclones of NIXT cells was prominently present on 5-25% of cells, but could leach off into the supernatant. The presence of this gp70 together with a receptor could serve as a constant auto-stimulatory blastogenesis signal to cause the uncontrolled lymphoblastic proliferation in the absence of leukemogenic MuLV's.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04802-06-LVC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cocarcinogenesis Studies of Mammary Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of these studies is to generate and characterize novel variants of murine mammary tumor viruses (MMTVs) and to employ these variants toward the development of in-vitro co-carcinogenesis transformation assays of murine and human mammary epithelial cells. Major accomplishments in this area during the last year include: (1) the selection of MMTV host range variants that have the ability to efficiently infect cells productively in-vitro; (2) development of cell substrates for mammary cell infectivity and transformation assays: (a) refined culture techniques have been established for the propagation of primary and secondary cultures of murine, rat, and human mammary epithelial cells and (b) cloning and characterization of cells have been obtained from cell lines derived from normal murine mammary glands; (3) MMTV host range variants have been utilized in combination with chemical carcinogens and tumor promotors, to develop in-vitro transformation assays for mammary epithelial cells.

Project Description

OBJECTIVES: To generate and characterize novel variants of murine mammary tumor viruses (MMTVs) that have the ability to infect a wide range of cells in-vitro and to employ these variants toward the development of an in-vitro co-carcinogenesis assay for the transformation of mammary epithelial cells. Specifically, we intend to:

1. Select for MMTV host range variants, derived from several mouse strains with high mammary tumor incidence, that have the ability to efficiently infect cells productively in-vitro. Emphasis will be placed on variants that have the ability to efficiently infect murine, rat, and human cells, respectively, in-vitro.

2. Characterize individual host range variants with respect to (a) structural proteins and antigenic determinants employing monoclonal antibodies, (b) RNA genomes and proviral DNA employing liquid hybridization and restriction mapping, (c) oncogenicity, and (d) host range tropisms in-vitro.

3. Develop cell substrates for mammary cell infectivity and transformation assays: (a) refine culture techniques for the propagation of primary and secondary cultures of murine and rat mammary epithelial cells (human primary cell culture techniques are being done collaboratively) and (b) clone and characterize cells obtained from cell lines derived from normal murine mammary glands.

4. Employ MMTV host range variants in combination with chemical carcinogens and tumor promoters to develop in-vitro co-carcinogenesis transformation assays in rodent and human mammary epithelial cells.

BACKGROUND AND RATIONALE: MMTVs are the only mammalian retroviruses known to induce carcinomas. An understanding of the mechanism by which MMTVs and related viruses are involved in the etiology of mammary cancer, however, has previously been hampered by both (a) the inability of MMTVs to infect cells in-vitro, and (b) the lack of suitably characterized cell substrates of normal mammary epithelial cells of any species. Our laboratory has recently demonstrated that the highly oncogenic MMTVs of C3H, RIII, and GR mice could infect both feline and mink cells. Establishment of these productive infections, however, required extremely high multiplicities of infection (moi) (as high as 5×10^{11} particles were required to infect 10^6 cells) and long latent periods for the synthesis of de-novo MMTV (up to ten weeks). We have previously shown that various MMTVs synthesized by feline or mink cells in culture retain their antigenic reactivities in group-specific radioimmunoassays for MMTV virion proteins and have a polypeptide profile similar to that of wild-type MMTVs. These stocks of MMTVs also exhibited no evidence of either murine or feline type-C viruses as determined by radioimmunoassay for type-C retrovirus p30 antigens, electron microscopy, gel electrophoresis of virion proteins and assays for virion-associated DNA polymerase activity.

The rationale of the studies reported here was to isolate host range variants of MMTVs that have the ability to efficiently infect a broad

range of cells in-vitro. These host range variants could then be used (a) to elucidate, at a molecular level, the mechanisms of MMTV replication and/or transformation, (b) in the development of a well defined in-vitro assay for the transformation of mammary epithelial cells, and (c) as reagents in immunologic and nucleic acid studies in distinguishing viral from host cell determinants.

MAJOR FINDINGS:

We have previously demonstrated that MMTVs from GR, RIII, and C3H mice can productively infect feline and mink cells in culture, but progeny viruses were not released until up to ten weeks after infection. We have used the MMTVs released by feline CrFK cells, designated MMTV(C3H)vp1, MMTV(RIII)vp1, and MMTV(GR)vp1, to infect other CrFK cells; the term "vp" denotes virus passage number. A dramatic reduction (as much as 50,000-fold) in the moi required to infect feline cells is observed with MMTV vp1 preparations, as compared to wild-type MMTVs obtained from murine cells. The viruses produced by feline CrFK cells infected with vp1 preparations were designated MMTV(C3H)vp2, MMTV(RIII)vp2, and MMTV(GR)vp2, respectively. These latter three viruses were then used to infect new CrFK cells; as few as 10^2 MMTV(C3H)vp2 particles resulted in a productive infection. Several experiments have also demonstrated a significant shortening of the latent period to the synthesis of de-novo virus, for any given moi, as a result of serial virus passage in these cells. Whereas progeny virions were not synthesized until as late as 90 days post infection of CrFK cells with wild-type MMTV(C3H), MMTV(C3H)vp3 productively infected CrFK cells with a latent period of as short as four days. Further serial passage of this virus up to five more times (a total of eight serial virus passages), however, did not result in any further significant shortening of the latent period to de-novo progeny virus synthesis. It has previously been shown that both insulin and dexamethasone stimulate the synthesis of MMTV in mouse mammary tumor cells and CrFK cells. A 3-fold stimulation in the synthesis of MMTV host range variants is observed when insulin is added to cell culture medium. When dexamethasone is added, however, either with or without the presence of insulin, we have observed a 15-fold stimulation of virus production.

Since MMTV host range variants are of potential use in in-vitro transformation assays, the in-vivo oncogenic potential of these viruses was determined. BALB/c mice were chosen as the host due to their previously reported susceptibility to MMTV replication and oncogenicity. An increase in mammary tumor incidence was observed over inoculation doses ranging from 10^5 to 10^8 wild-type particles. A similar degree of oncogenic potential was also observed employing the MMTV(C3H)vp4 variant as inoculum, i.e., an increase in the percent mammary tumors was observed over a wide range of doses. Inoculation of as little as 10^4 MMTV(C3H)vp4 particles resulted in 41% mammary tumors. These tumors were shown to contain de-novo proviral sequences specific for the inoculating virus.

The MMTV host range variants have been developed and assayed for their ability to productively infect a variety of cell substrates. Twelve cell lines from six species including feline, murine, canine, rat, bat and human were susceptible to productive infection. As expected, variability between cell lines in latent period to the appearance of de-novo virus was observed. Some cell lines exhibited restriction of growth of one variant while being permissive to replication of the other variant. Secondary rat embryo cultures, for example, were permissive to infection with the RIII variant but restrictive to the growth of the C3H variant. Conversely, the bat lung cell line, TblLu, was permissive to infection by the C3H variant but totally restrictive to the growth of the RIII variant.

We have also conducted experiments on the relative susceptibility to infection by MMTV of cells following prolonged passage in culture. In a period of continuous culture extending more than one year, we have not seen any change in the susceptibility of CrFK cells to infection by MMTV host range variants. A variety of cell lines including NIH-3T3, Cf2Th, FRE, and TblLu similarly did not exhibit any change in their susceptibility in a period of extended culture lasting more than one year. On the other hand, FeF feline cells at passage 17 were readily susceptible to infection, but the same cells at passage 152 were refractive to productive infection. We have obtained similar results with both the RIII and the C3H MMTV variants. FeF cells (early vs. late passage) therefore provide an excellent system to analyse those factors that may influence permissiveness to MMTV productive infection.

The maximum titers of progeny virus obtained after infection of cells with MMTV variants varied from 7.1×10^5 to 1.2×10^8 particles/ml/24 hrs. In any one cell line permissive to the replication of both viruses, however, the titers rarely differed by more than two-fold.

We have recently serially passaged both the RIII- and C3H-variants in NIH-3T3 cells in an attempt to enhance the infectivity of these viruses for murine cells. Serial passage of the RIII variant up to three times in murine NIH-3T3 cells reduced the latent period to the synthesis of progeny virus for any given moi. Infection of murine cells with MMTV(RIII)vp4 at an moi of 1×10^3 resulted in the appearance of de-novo virus after a latent period of 34 days. When this progeny was then used to infect other cultures of NIH-3T3 cells at an moi of 1×10^3, the latent period was reduced to 12 days. In a similar manner, we have serially passaged MMTV(RIII)vp4 in rat cells (FRE), canine cells (Cf2Th) and human placenta cells (HP574). In each case, serial virus passage resulted in a reduction in the latent period to the appearance of de-novo progeny virus, for any given moi. Serial virus passage of MMTV(RIII)vp4 in rat cells resulted in a reduction in the latent period from 30 to 40 days to only six days, at an moi of 1×10^4. In all cases, moreover, serial virus passage did not result in any reduction in the efficiency with which these new variants productively infected feline cells.

All MMTV host range variants retained their antigenic reactivities in group-specific radioimmunoassays for both MMTV gp52 and p28. All MMTV variant stocks were negative when tested for type-C viral antigens.

The preparation and characterization of primary cell cultures from normal mammary glands (NMG) of rats has been previously described. Briefly, partial collagenase digestion and Ficoll gradient sedimentation facilitated the separation of epithelial elements derived from mammary glands of perphenazine stimulated normal virgin rats. In addition to morphological features, the rat NMG cultures were characteristic of mammary cells in that they produced alpha-lactalbumin as detected by specific radioimmunoassay. Rat NMG cells examined in our laboratory contained desmosomes, tonofibrils, and microvilli, further indicating their epithelial nature. Cultures from normal mammary glands of Wistar-Furth, Wistar-Lewis, ACI, and F344 (Fischer) rats were all permissive for productive infection by MMTV host range variants. MMTV-infected rat NMG cultures were maintained up to three months with consistent virus production.

Cultures of two cell lines established from normal mammary glands of mice were used as substrates for MMTV host range variant infections; these were the Namru line (NMuMG) of Owens and the C57MG line of Vaidya and Lasfargues. Both cell lines were permissive to productive infection by both the MMTV(RIII)vp4 and the MMTV(C3H)vp4 variants with a direct relationship between the dose of inoculating virus and the time to the appearance of de-novo virus.

The C57BL/6 mammary gland cell line, C57MG, was cloned at passage 31. Single cell suspensions of the uncloned and cloned C57MG cell lines were inoculated into both syngeneic C57BL/6N and athymic (nude) mice (2×10^6 cells/mouse). In three separate experiments, the uncloned C57MG parent cell line and each of the clones failed to induce tumors in either syngeneic mice or athymic (nude) mice during observation periods of as long as 154 days. C57MG single cell clones were tested for permissiveness to infection with the MMTV(RIII)vp4 host range variant. Clone 2 cells were readily productively infected by this virus and have continued to produce progeny virus for three months. Clone 1 cells produced virus transiently for the first two weeks after infection and thereafter virus production was not detectable. In contrast, clone 3 cells were totally restrictive to productive infection.

We have used clone 2 of the C57MG line as a substrate for viral and chemical transformation studies. This cell line has the following properties: the cells are of murine origin as determined by karyotype analyses, isoenzyme analyses and immunofluorescence using a variety of species-specific antisera; they contain desmosomes, secretory granules, and casein mRNA; the cells metabolize 7,12-dimethylbenz(a)anthracene; and the phorbol ester tumor promoting agent, 12-tetradecanoyl phorbol-13 acetate (TPA) is not toxic to these cells. We have infected cultures of

these cells with MMTV(RIII)vp4 and subsequently treated them with 1 and 5 ug/ml DMBA and with 25 ng/ml TPA. In preliminary studies, we have noted morphological changes as a result of treatment of cells with virus and carcinogen. Cells either infected with virus or treated with carcinogen resemble untreated cells. Cultures receiving both virus and chemical carcinogen have an altered morphology showing increased random orientation of cells and much reduced cell-to-cell contact. The appearance of grossly abnormal looking cells with rough cytoplasmic membranes, long pseudopodia and increased nuclear to cytoplasmic ratios is also seen. Cultures of these morphologically altered cells are now being assayed for in-vitro transformation markers and have been inoculated into athymic (nude) mice. Treatment of cells with MMTV, DMBA or both agents resulted in approximately a 2-fold increase in colony formation in soft-agar. The most striking alteration in the phenotype of cells exposed to both virus and carcinogen was a dramatic increase (approximately 10-fold) in their tumorigenic potential in nude mice. This latter change in phenotype occurred at the same time as the development of morphological alterations in cells exposed to both agents.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROPOSED COURSE: The generation of host range variants of MMTVs has now made possible the study of the molecular events involved in the replication of, and in-vitro transformation by, mammary tumor viruses. Isolation of individual variants that have the ability to efficiently infect murine, rat and human cells, respectively, now also makes possible their use in in-vitro co-carcinogenesis experiments to demonstrate transformation of cells in each of these species. Furthermore, by obtaining MMTVs grown in heterologous cells, ambiguities concerning viral versus host cell elements can finally be vastly reduced or eliminated. These MMTVs have thus proved extremely valuable in the restriction endonuclease and monoclonal antibody studies.

There has been much conjecture about whether the process of transformation from normal to malignant cells progresses by way of a premalignant cell population. However, since these experiments were not conducted with defined cloned cells, it is difficult to delineate whether this is a multi-step process deriving from a single cell, or is due to quantitative changes in growth rates of different cell populations. It is anticipated that the MMTV host range variants, with demonstrated oncogenic potential and high efficiency of infection of mammary cell substrates, may now be successfully employed, either alone or in combination with chemical carcinogens and tumor promoters, in in-vitro transformation studies to provide answers to this question. Finally, the existence of cloned mammary cell lines with normal phenotypic properties, and a transformed counterpart, would be invaluable in the identification of those genomic elements involved in mammary cell transformation, as well as the identification of specific proteins either expressed on the cell surface, or intracellularly, that are involved in mammary cell transformation. The techniques of restriction endonuclease mapping of host cell genomes and

the use of monoclonal antibodies to detect unique antigenic determinants (both employed in our laboratory) are sufficiently sensitive and specific to identify such markers if they exist.

PUBLICATIONS:

Howard, D. K. and Schlom, J.: Differential host ranges for in vitro infectivity of mouse mammary tumor viruses. J. Gen. Virol. 47:439-448, 1980.

Schlom, J., Howard, D. K., and Horan Hand, P.: In vitro infection of mammary and non-mammary cells by mammary tumor viruses. In McGrath, C. M., Brennan, M. J., and Rich, M. A. (Eds.): Cell Biology of Breast Cancer. New York, Academic Press, Inc. (In Press).

Schlom, J., Colcher, D., Drohan, W., Horan Hand, P., Howard, D., and Teramoto, Y. A.: Systematics of murine mammary tumor viruses. In Brennan, M., Rich, M. A., and McGrath, C.M. (Eds.): Advances in Breast Cancer Research. New York, Academic Press, Inc. (In Press).

Howard, D. K., and Schlom, J.: Isolation of a series of novel variants of murine mammary tumor viruses with broadened host ranges. Int. J. Cancer (In Press).

Schlom, J.: Type-B and type-D retroviruses. In Stephenson, J. (Ed.): Molecular Biology and RNA Tumor Viruses, Chapter 11. New York, Academic Press, Inc., 1980, pp. 447-484.

Schlom, J., Drohan, W., Teramoto, Y. A., Young, J. M., and Horan Hand, P.: Diversity of mammary tumor viral genes and gene products in rodent species. In Cold Spring Harbor Conferences on Cell Proliferation - Viruses in Naturally Occurring Cancer, Vol. 7, (In Press).

Schlom, J.: Viruses and breast cancer. In Strax, P. (Ed.): Control of Breast Cancer Through Mass Screening. Massachusetts, PSG Publication Co., Inc. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04812-12-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Endogenous type B viruses and cell components regulating cell division and configuration

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	U.I. Heine	Research Microbiologist	LVC NCI
Other:	R.G. Suskind	Medical Director	LVC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.75	0.75

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study was initiated to investigate the role of viral and cytoskeletal proteins in the maturation and assembly of endogenous, type B retroviruses of murine origin. Intracytoplasmic type A particles, precursors of such viruses, are associated with microtubule organizing centers (MOTC), the mitotic apparatus and microtubules per se. By arresting cells at various stages of mitosis by Colcemid and vincristine sulfate, we could show that the migration of such precursor particles from the cytoplasm to the kinetochores of the chromosomes is a function of the inhibition of microtubule formation at the pericentriolar cytocentrum as well as the inhibition of spindle fiber formation at chromosomes. The association of type A particles with the mitotic apparatus is reversible by removal of the inhibitor and is inversily proportional to the reattachment of spindle fibers to chromosomes. The active participation of microtubule and cytoskeletal proteins in the intracellular transport and maturation of type B viruses was confirmed by immunoelectron microscopic studies of of the distribution of anti-tubulin and anti-actin in virus infected cells.

PHS-6040
(Rev. 10-76)

Project Description

Objective:

To identify the precise nature of and molecular basis for the interaction of endogenous retroviruses with microtubule organizing centers and cytoskeletal elements of the infected cell.

Methods Employed:

Tissue culture techniques, virus propagation and assays, light and transmission electron microscopy, immunomicroscopic techniques at the level of the light and electron microscoope.

Major Findings:

Recent electron microscopic studies have shown that certain endogenous type B tumor viruses in hamster and murine species have precursor particles (intracytoplasmic type A particles) that are found in association with microtubule organizing centers at the centriole in interphase cells and at the kinetochore region of metaphase-arrested chromosomes. The cytoskeleton, consisting of microtubule organizing centers (MTOC), of microtubules, centrioles and kinetochores is determinative both in regulating cellular configuration in interphase cells and in the formation of the mitotic spindle during cell division. During mitosis, for example, duplicated chromosomes must be divided equally among daughter cells. To accomplish this precise distribution of the genetic material, centriole and DNA synthesis must be coordinated during the cell cycle. The importance of the involvement of retroviruses with the cytoskeleton has been stressed by recent reports that a protein kinase coded by the viral gene sarc may activate (phosphorylate) a protein of the cytoskeleton (Collet and Erikson, PNAS 75:2021, 1978; Sen and Todaro, Cell 17:347, 1979). Interference of viral precursor particles with such central cell organelles as are combined in the cytoskeleton may effect their function and may influence cell mitotic behavior as well as the functional role of the microtubule organizing centers.

An association of microtubules, especially those of the mitotic apparatus, with the assembly of reoviruses has been reported by Dales et al. (Virology, 25:193, 1965). However, the possible wider involvement of these organelles in virus assembly and transport has not been considered until recently. Wheatly (J. Gen. Virol. 24:395, 1974) observed the presence of intracytoplasmic type A particles a the centriolar region of Chinese hamster ovary (CHO) cells and suggested that this observation might be relevant to the replication and epigenetic vertical transmission of endogenous tumor viruses. Also, studies of the nucleation of microtubule formation revealed the constant association of intracytoplasmic virus-like particles with sites of microtubule formation near centrioles and kinetochoes in CHO cells (Gould and Borisy, J. Cell Biol. 70:43a, 1976) and PtK1 cells (Pepper and Brinkley, Chromosome 60:223, 1977). These particles were found to be similar to type A particles of retroviruses

that in other species are known to be precursors to retroviruses of type B and D, i.e., the mammary tumor agent, the Mason-Pfizer virus, and the guinea pig virus. In CHO and PtK, cells, however, mature virions are not yet known. We recently described that type A particles of three endogenous retroviruses rescued from the outbred South-East Asian mouse are also associated with different parts of the cytoskeleton (Heine and Todaro, 1978). Since, in these cases, precursor particles as well as mature virions exist side by side we have obtained an excellent model to study the interplay between endogenous type B viruses and the host cell's mitotic apparatus and chromosomes in the assembly and transmission of such viruses. To clarify this relationship, the localization of both viral precursor particles as well as cytoskeletal proteins, such as tubulin and actin, and tumor virus antigens is under study using techniques of whole mount electron microscopy of chromosomes and immunomicroscopy at both the light and electron microscope levels in addition to standard electron microscopic examinations. Three new virus isolates, M432, M832 and M960, obtained from the South East Asian mouse have been characterized morphologically in ultrathin sections and by negative staining with respect to their composition and intracellular assembly. The three isolates share certain morphological properties with the murine mammary tumor virus, a retrovirus of type B. In the three classes of viruses intracytoplasmic particles of type A are precursors of the mature virons. In interphase cells numerous complete type A particles (doughnut forms) and incomplete type A particles of crescent shape can be observed in close association with the microtubule organizing center and the centriole, all being embedded in the matrix of the cytocentrium. Only few type A particles are scattered throughout the cytoplasm, always being in close apposition to microtubules, or adjacent to the cell membrane, where they are released from the cell by budding. In metaphase-arrested cells (0.1 ug/ml Colcemid, 5 hrs), the type A virus precursors accumulate at the chromosomes adjacent to the kinetochores at the initiation site of microtubules. In late stages of the mitotic cycle, dispersion of the type A virus precursors throughout the cytoplasm can be observed again, concomitantly with the reassembly of microtubules. Close association of type A viral precursors with microtubules is confirmed further by the attachment of the particles to paracrystals of tubulin which are formed in the cytoplasm after addition of the microtubule inhibitor vincristine sulfate to the tissue culture medium.

These observations, for the first time, give evidence that virion belonging to the family of Retroviridae can be related to microtubule and MTOC-associated virus precursors, thus being closely associated with the centriole in interphase cells and the kinetochore-plate at the centromere region of chromosomes in metaphase-arrested cells.

These findings make feasible an investigation in the relationshp of intracytoplasmic type A particles to the cytoskeleton. Briefly, we have previously demonstrated the close association of microtubules and microtubule proteins with the maturation and transport of endogenous virus particles of type B. However, the significance of these observations in the cell biology of tumor virus infection remains obscure partly because immunomicroscopic

techniques at the level of the electron microscope such as immunoferritin and immunoperoxidase techniques have not been convincing at other than cell surfaces. Recently, however, methods of fixation have been developed that can control the intracellular localization of immunoglobulins (Willingham and Yamada, J. Histochem. Cytochem. 27:947, 1979). By employing these methods, we have been studying the distribution of anti-tubulin and anti-actin in murine cells carrying type A viral precursors of strain M432. With anti-tubulin, we were able to specifically stain microtubules and MTOC as well as intracytoplasmic type A particles when they were located near these cellular organelles. Extracellularly, mature type B virions did not react with the immunoglobulin. Staining with anti-actin could also be considered as being highly specific in our experiments, due to its confined localization adjacent to the cell membrane and in microvilli, both being known for their high actin concentration. The association of type A particles with actin, however, is still under investigation. Studies regarding the localization of viral internal proteins (P24, P28) in the cell have just been initiated and results are not yet available.

Significance to Biomedical Research and the Program of the Institute:

Although there have been recent fundamental advances, both in the understanding of the organization of the viral genome of retroviruses, as well as of the interaction at membranous cell surfaces, little is known about morphologic as well as immunologic events in the assembly and maturation of such viruses in the cytoplasm. Our studies have demonstrated the involvement of the cytoskeleton, i.e., microtubule organizing centers, microtubules, centrioles and mitotic apparatus, in the assembly and maturation of precursor particles of type B retroviruses.

Proposed Course:

The recent development of fixation methods that control the intracellular localization of immunoglobulins permits studies regarding the localization of viral proteins (P24, P28) in the infected cell. Such studies are now under progress in the laboratory.

Publications:

Heine, U.I., Kramarsky, B., Wendel, E. and Suskind, R.G.: Activation of endogenous virus in Chinese hamster cells associated with microtubules and the mitotic apparatus of the host cell. J. Gen. Virol. 44:45-55, 1979.

Heine, U.I., Margulies, I., Demsey, A.E. and Suskind, R.G.: Quantitative electron microscopy of intracytoplasmic type A particles at kinetochores of metaphase chromosomes isolated from Chinese hamster and murine cell lines. J. Gen. Virol. 45:631-640, 1979.

Heine, U.I., Suskind, R.G., Margulies, I. and Demsey, A.E.: Association of type B retroviruses with the cytoskeleton. 2nd International Congress on Cell Biology (1980) (in press).

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Schafer, M.P., Rohrmann, G., Heine, U. and Beaudreau, G.S.: DNA from two orgyia pseudo tsugata baculoviruses: molecular weight determination by means of electron microscopy and restriction endonuclease analysis. Virology 95, 176-184, 1979.

Gogusev, J. and Heine, U.: Etude ultrastructural du genome du virus de la myelocytomatose aviaire (MC29). C.R. Acad. Sc. (Paris) 287, 1075-1078, 1978.

Sherwin, S.A., Bonner, T.I., Heine, U. and Todaro, G.J.: The isolation of an endogenous retrovirus from the new world rodent Dasyprocta punctata (agouti). Virology 94, 409-416, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04814-09-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunomicroscopic and E.M. Studies Concerning the Intracellular Localization of MTV Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Suskind	Medical Director	LVC NCI
Other:	U.I. Heine	Research Microbiologist	LVC NCI
	A. Demsey	Expert	LVC NCI
	P.H. Levine	Medical Director	LVC NCI
	J. Schlom	Microbiologist	LVC NCI

COOPERATING UNITS (if any)
Laboratory of Experimental Pathology, NIAMD

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An immunomicroscopic and electronmicroscopic study is being conducted to localize viral antigens of endogenous type B and D retroviruses during cell division, particularly in cell cultures heterologously infected with mammary tumor virus. Clinical and laboratory aspects of the relationship between human mammary carcinomas and MTV antigens are also under study.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

Evidence for the possibility that cytoskeletal proteins, such as actin and tubulin may be involved in the transport or release of endogenous type B and type C tumor viruses has accumulated in several laboratories (C.H. Damsky et al., J. Cell Biol. 75:593-605, 1979; A. Sen, G.J. Todaro, Cell 17:347-356, 1979, Proc. Nat. Acad. Sci., in press). Previous electron microscopic studies have shown that certain precursor particles of endogenous type B tumor viruses are associated with microtubule organizing centers at the centriole in interphase cells and at the kinetochore region of metaphase arrested chromosomes. The association of these particles with the mitotic apparatus has attracted interest as a possible modality of epigenetic transmission. In order to determine a possible wider significance of these observations in the assembly and transmission of type B retroviruses we are attempting to (1) localize intracellular murine mammary tumor virus antigens in infected cell cultures of homologous and heterologous species, (2) to determine whether these can be related morphologically or immunologically to the mitotic apparatus, (3) to characterize the relationship of cytoskeletal proteins to viral precursor particles and viral antigens, (4) to investigate a possible relationship between mouse mammary tumor viral antigens and mammary carcinomas of heterologous species including man.

Methods Employed:

Immunomicroscopic procedures at the level both of the light- and electron microscope, such as various immuno-peroxidase, and immunoferritin procedures have been adapted and employed in combination with recently developed methods of fixation that allow the intracellular penetration of immunoglobulins (M.C. Willingham, S.S. Yamada, J. Histochem. Cytochem. 27:947-960, 1979). The intracellular localization of mammary tumor virus antigens has been studied in a feline cell line (CrFK) continuously infected with a host cell variant of MMTV (C3H) (D.K. Howard & J. Schlom, Proc. Nat. Acad. Sci. USA, 74:5718-5722, 1978) using antisera both to whole virus, as well as several monoclonal antibodies from murine hybridomas (prepared and supplied by Dr. Schlom). The characterization by radioimmune precipitation of these monoclonal antisera has been initiated in collaboration with Dr. Demsey.

Major Findings:

Immunomicroscopic techniques have been developed that permit the intracellular localization of MTV antigens without admixture of murine cellular components and fixation techniques permitting the localization of such antigens at other than cell surfaces and membranes have been adaped.

Using a peroxidase-antiperoxidase technique at the level of the light microscope, it has been shown that in cells fixed with low concentrations of either paraformaldehyde or glutaraldehyde, reactivity to purified,

disrupted, whole virus antibody can be demonstrated not only at the cell surface of interphase cells, but that the mitotic apparatus is reactive in dividing cells. Preliminary results indicate a variation of peroxidase staining within phases of the mitotic cycle. During late prophase and early metaphase perichromatin staining is observed, whereas in late metaphase and anaphase the region of the mitotic apparatus is primarily reactive. During telophase diffuse reactivity is spread throughout the cytoplasm, but the reactivity of the primary cleavage furrow is accentuated. The extent to which cytoskeletal proteins, physically associated with purified virus preparations used as antigen, or specific viral proteins may contribute to this phenomenon is not clear at present. Current investigations are concerned with clarifying this by using monospecific and monoclonal antibodies in immunomicroscopic studies at the level of both the light and electron microscope. In collaboration with Dr. Demsey several such monoclonal antibodies (developed in Dr. Schlom's laboratory) of MTV isolates from Asian feral mouse species murine hybridomas have been initially characterized by radioimmunoprecipitation and have been shown to contain antigenic determinants coincident with gP52, gP36 and also with p28 internal protein. Using various bridge procedures with appropriate secondary antisera initial results indicate a similar reactivity of some monoclonal IgG_1 antibodies in light microscopic immunoperoxidase assays.

Electron-microscopically, it has been shown that MTV-infected CrFK cells contain intracytoplasmic type A precursor particles, however without recognizable affinity to the centriole or mitotic apparatus. Fixation procedures that permit intracellular penetration of immunoglobulins (M.C. Willingham and S.S. Yamada, J. Histochem. Cytochem. 27:947-960, 1979) are being applied to electron microscopic immuno-ferritin and immunoperoxidase techniques in parallel with immunomicroscopy. In incomplete experiments only labeling of budding virus particles and intracytoplasm type A particles have been obtained so far.

Related studies are being conducted in collaboration with the Clinical Studies Section, in order to confirm and explore the presence of an immunologic cross-reaction between the group specific gP 52 antigen of murine MTV with invasive human mammary carcinoma in high incidence, using an indirect immunoperoxidase procedure (R. Mesa Tejada, I. Keydar, et al., Proc. Nat. Acad. Sci. USA, 75:1529-1533, 1978). A population of breast cancer patients in Tunisia, with a high incidence of a rapidly progressing form of this disease ("inflammatory carcinoma") is being used in an attempt to confirm such a finding. Initial collaborative studies performed at the Institute of Cancer Research, Columbia University, with antisera developed at that laboratory and show that about 90% of these cases exhibit a positive immunoperoxidase reactivity with either of two MMTV antisera. This reactivity appeared unrelated to clinical aggressivity. Whereas we have found some evidence that these results can be reproduced with a small sample of antiserum (supplied by Dr. Keydar), we have so far not been successful in reproducing similar results with other reagents, except in murine mammary carcinomas known to contain MTV. We are, however, continuing such an effort, especially with monoclonal

antisera containing heterospecific antigenic determinants for gP 52 (supplied by Dr. Schlom). We have also failed to find evidence of cross reactivity with antiserum to whole disrupted MMTV (C3H) in some canine mammary tumors (supplied by Dr. Fritz, Argonne National Laboratory).

Significance to Biomedical Research and the Program of the Institute:

Immunomicroscopic techniques both at the level of the light and electron microscopes suitable for studying the intracellular localization of viral antigens and their interaction with cytoskeletal protein have been adapted and developed. Initial observations suggest a relationship of endogenous tumor virus antigens to spindle attachment and microtubule dissemination during the mitotic cycle. These studies should be of relevance to an understanding of the cellular biology of transformation and suggest the possible participation of an epigenetic mode of transmission of some tumor viruses. A characterization of heterospecific determinants of mammary tumor virus antigens may be of pathogenetic or etiologic significance in the development of human mammary carcinoma.

Proposed Course:

Having established suitable methods of fixation and bridging procedures with light-microscopic immunoperoxidase techniques we propose to apply these to immunoferritin techniques in the electron microscope and pursue therewith the outlined objectives of this project. These will include also the search for an localization of heterospecific determinants of MTV in human cell lines and mammary carcinoma. The possibility of extending similar immunomicroscopic procedures to Epstein-Barr virus and cytomegalovirus associated tumors will be explored.

Publications:

Heine, U.I., Kramarsky, B., Wendel, E. and Suskind, R.G.: Activation of endogenous virus in Chinese hamster cells associated with microtubules and the mitotic apparatus of the host cell. J. Gen. Virol. 44:45-55, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04821-08-LVC

PERIOD COVERED: October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Strategies for the Isolation of Tumor Viruses from Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

None

LAB/BRANCH: Laboratory of Viral Carcinogenesis

SECTION: Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were made concerning the natural mechanisms for transmission perpetuation and evolution of type C viruses and virogenes in mammals, especially primates. The mechanism of expression and regulation of these genes has been explored with the identification and preliminary characterization of several peptides and factors which effect phenotypic changes typical of malignancy. Several new primate and murine endogenous viruses were isolated from Asian mice and primates closely related to humans. These primate viruses will allow the development of specific reagents with which human cells may be examined for the expression of viral related genetic material.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To develop new strategies and methodology, or to adapt existing approaches, for the detection, isolation, and characterization of oncogenic viruses associated with primates. This includes virus-related genetic sequences detectable in whole or in part in the nucleic acids of normal or malignant cells derived from primates, including the human.

Methods Employed:

Increasing numbers of retroviruses (both endogenous and infectious) have been isolated from tissues and cell lines of primates using cocultivation techniques. Type C and type D viral isolates have been obtained from several species of Old and New World monkeys, including woolly monkeys, gibbons, baboons and langurs. These viruses can be used for nucleic acid hybridization studies which reveal their origins and modes of transmission, including interspecies (or trans-species) infection. Genetically transmitted viral genes have been identified in both New World and Old World monkeys. When whole virions were isolated they have been classified as type C or type D viruses based on morphologic properties. They were further subdivided into several distinct classes according to both antigenic and nucleic acid hydridization criteria.

Major Findings:

There are certain primate species from which a particular class of endogenous retrovirus is isolated with high frequency (baboons among the Old World primates and squirrel monkeys among the New World primates). There are other groups of animals from which endogenous retroviruses have been obtained by similar methods, but only at low frequency. The probability of virus isolation, therefore, does not appear to be related to the number of endogenous DNA copies detected or to the levels of viral-specific RNA expressed. Both the low probability of isolation and the very long period needed to detect virus replication suggest that still other primate species will yield endogenous viruses. These may be genetic relatives of the viruses previously isolated or may represent members of some as yet undetected retroviral classes. It also appears that a majority of primate species are capable only of low frequency virus activation.

Three new viruses, isolated from primates closely related to man (langur, owl and stumptail monkeys) were biochemically and immunologically characterized. They are endogenous, genetically transmitted viruses; thus the viral information was detectable in the DNA of normal cells. It was demonstrated that related species have related viral genes in their DNA and that those gene sequences have evolved with the species and are contained in the DNA of

both New World and Old World primates. These isolates have brought the total of genetically transmitted primate viruses to 5 (3 type C and 2 type D).

None of the new type C viruses are closely related by hybridization or by immunologic parameters to the previously described baboon type C viruses or to the squirrel monkey endogenous type D viruses. A single Old World monkey species, for example, the African green monkey, can be shown to contain multiple copies of at least three distinctly different retroviral classes (two type C and one type D) in its normal cellular DNA. Cell lines in culture that are not releasing virus can be shown, nevertheless, to be transcribing viral-specific RNA. A library of green monkey cellular genes cloned in PBR322 made available by Dr. Maxine Singer, NIH, are being screened in order to obtain the cellular virogene sequences for each of these viruses.

The fact that viruses have been isolated from man's closest relatives and that related DNA sequences have been detected in human DNA, suggests that as technology improves and experience is gained, other primate species, including man, will most likely yield these types of endogenous oncogenic viruses. The existing isolates have made possible the development of biochemical and immunological assays for markers related to viral gene expression in a variety of human normal and malignant tissues. Each new virus isolate allows the development of specific reagents (nucleic acid probes, antigens, enzymes) with which human populations may be examined for the expression of viral genetic material.

Two additional new type C viruses were isolated from mice originating from Southeast Asia. The unusual finding concerning these isolates was their demonstrated preferential relationship to primate type C viruses (woolly monkey and gibbon ape) rather than to the classical murine type C oncogenic viruses. The fact that endogenous viruses are genetically transmitted in animal species has served to demonstrate the evolutionary progression of oncogenes in the various species, including primates and man, and how interspecies transmission and recombination of oncogenic viruses may occur.

Significance to Biomedical Research and the Program of the Institute:

This work is significant because it has provided a sound basis for the understanding of the interaction between environmental and genetic aspects of cancer etiology, susceptibility, and resistance. The number of classes of different viruses carried as mouse genes is extensive. Two families of type C viruses and two families of type B viruses are carried in the genetic material of all species of mice. A similar situation appears to be the case with primates. Further isolations of additional endogenous primate retroviruses, including perhaps those of apes and man, may be feasible and therefore require the development of new technology as well as the continuation of current attempts for prolonged cocultivation with unusually permissive host cells.

Table 1

Origin and Transmission of Primate Retroviruses

Virus	Isolation	Endogenous, in Normal DNA of Host	Origin
Gibbon Woolly Monkey	(GaLV, G-Br, SEATO, SSAV)	No	Mice (Mus spp.)
Baboon	Baboon tissues and cell lines (M28, M7, PP-1, etc.)	Yes	Baboons
MPMV and	Rhesus tissues	No	Langurs
Langur	Langur cell line (PO-1-Lu)	Yes	Langurs
Squirrel monkey	Squirrel monkey tissues and cells (SMRV, M534, M706, etc.)	Yes	Squirrel monkeys
Owl monkey	Owl monkey kidney (OMC-1)	Yes	Owl monkeys
Macaque	Stumptail monkey spleen (MAC-1)	Yes	Macaques
Colobus	Colobus (CPC-1) monkey	Yes	Colobus monkey

Proposed Course:

This project will continue as described.

Table 2

Primate tissues tested for their ability

to release infectious retroviruses

Primate	No. of Tissues or Cell Cultures Tested	No. of Positive Isolates	Percentage Positive
Old World Monkeys			
Cercopithecinae			
Papio (baboon)*	22	17	77
Theropithecus (gelada)*	7	4	57
Cercocebus (mangabey)	10	0	0
Erythrocebus (patas)	7	0	0
Ceropithecus (vervet)	24	0	0
Macaca (macaque)	65	1	2
Colobinae			
Colobus (colobus)	18	1	6
Presbytis (langur)	12	1	8
New World Monkeys			
Saimiri (squirrel)*	17	14	82
Lagothrix (woolly)	8	0	0
Callithrix (marmoset)	16	0	0
Ateles (spider)	12	0	0
Aotus (owl)	9	1	1

*These three species yield retroviruses readily during cocultivation and can be classified as "high frequency" virus releasers.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04822-10-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Application of Virologic Tools to Control of Viral-Associated Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	C.W. Berard	Pathologist	LP NCI
	R. Biggar	Medical Officer	LVC NCI
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LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Clinical Studies Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is to identify human tumors associated with specific viruses and to apply virologic tools to studies on the etiology, prevention, early diagnosis and control of these malignancies. Current projects include: studies on the relationship of Epstein-Barr virus (EBV) to nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL) in the United States; evaluation of EBV serology in the diagnosis and monitoring of patients with NPC, BL and other EBV related diseases; development of a primate model for BL; in vivo and in vitro tests for compounds with activity against EBV, and the identification of factors enhancing the dissemination of breast cancer.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

A. To utilize immunologic and epidemiologic techniques in studies on the etiology and control of EBV associated diseases, with particular emphasis on nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL).

B. To investigate the pathogenesis and treatment of rapidly progressing breast carcinoma (RPBC) in Tunisia as a model for aggressive breast cancer in the United States.

Methods Employed:

Project A

Pathology based registries were maintained for American NPC and BL as a source of cases for epidemiologic and laboratory studies. A battery of serologic tests were used to monitor patients with BL, NPC and other EBV-associated diseases. Marmosets and rhesus monkeys were inoculated with EBV and Herpesvirus Saimiri (HVS) and monitored by humoral and cell-mediated immunity (CMI) assays.

Project B

More than 100 Tunisian breast tumors from patients seen at the Institute Salah Azaiz (ISA) were studied by pathologic, biochemical and immunologic techniques to determine differences between RPBC and non-RPBC cases. Immunologic tests evaluated differences between Americans and Tunisians with and without breast cancer. Clinical and laboratory information on 104 patients entered into a chemotherapy study utilizing cyclophosphamide, methotrexate, and 5-fluorouracil were also analyzed.

Major Findings:

Project A

The IgA antibody to EBV VCA appeared to provide a useful diagnostic test for NPC. In a retrospective case-control study involving 70 NPC patients and a prospective multicenter NPC program, IgA antibody to VCA was significantly higher in the NPC patients than controls. A radiometric complement fixation (CF) assay showed that Greenland eskimos, who have a thirty-fold risk of developing NPC as compared to Americans, have significantly higher EBV antibody titers than Danes living in Denmark or Greeland. Danes living in Greenland were intermediate in titer. Demographic and pathologic information on more than 1000 newly diagnosed patients with NPC were reviewed, revealing a relationship between age, race, and histologic subtype. Lymphoepithelial tumors had a younger age distribution than either squamous or transitioned cell carcinomas. A bimodal pattern was seen for blacks, where teenagers were

affected more than young whites or Chinese. Prognosis was better for females and for young patients. Serologic studies indicated that patients with squamous cell NPC were less likely to have elevated IgA antibody titers to EBV VCA than patients with undifferentiated NPC.

Data from the American BL Registry revealed a correlation between age and target organs, indicating that BL affects the most rapidly growing tissues. Patients with high EBV titers had a better survival than patients with low titers.

Studies on EBV and HVS in vitro and in vivo indicated that marmosets and tissue culture cell lines could be utilized to study drugs and biologic modifiers capable of inhibiting EBV and HVS. A byproduct of the oncogenesis studies was the observation that interferon inducers can protect the bone marrow from radiation toxicity.

Project B

Hormone receptor studies demonstrated a correlation between progesterone receptor levels and a favorable response to treatment, but no differences between patients with and without RPBC were noted. Pathologic data supported the relationship between PEV-1 (non-inflammatory RPBC) and PEV with inflammatory signs; both groups had significantly poorer nuclear grade than PEV-0 tumors. Plugging of the dermal lymphatics was a frequent concomitant of PEV-2 and PEV-3. Immunologic studies demonstrated a higher general reactivity to a variety of antigens among normal Tunisians than normal Americans. RPBC patients had a vigorous immune response to tumor and virus-associated antigens whereas American breast cancer patients with advanced disease had suppressed immunity.

Significance to Biomedical Research and the Program of the Institute:

Project A

The availability of laboratory assays measuring immunity to EBV provides an opportunity to define the factors modifying the effect of EBV in different individuals. Additional diagnostic tools and predictors of morbidity may be developed from these new assays. The IgA antibody to EBV has proven to be an effective diagnostic test for NPC and is now being used frequently for patients in the Washington area. Studies on the interrelationship of pathology, age, racial/ethnic status and EBV serology will help to distinguish among cofactors in the studies on the etiology of NPC. The population based serologic studies in Greenland eskimos, Greenland Danes and Danish Danes may provide information on the relative importance of environment and genetics as related to the high incidence of NPC in eskimos. The American BL Registry is providing important information on BL in a non-endemic area, the United States, thus permitting studies on the pathogenesis of the disease without the complicating effect of malaria. Progress on the in vitro and in vivo studies of EBV and HVS will permit the testing of materials potentially useful in the control of EBV-associated tumors.

Project B

The development of a strong collaborative effort between the National Cancer Institute and the Institute Salah Azaiz in Tunisia is of great value in that it provides access to a group of patients with rapidly progressing breast cancer. Information gained as to the etiology and means of controlling this form of breast cancer in Tunisia can be expected to help in the control of fulminating breast cancer in the United States. The high frequency of RPBC in Tunisia allows information on this entity to be accumulated more rapidly than in the United States. Clinical and pathologic data to date indicates that the RPBC in Tunisia is no different than fulminating breast cancer in the U.S., and therefore information obtained from the Tunisian patients would be directly applicable to breast cancer patients in the United States. The finding of the high content of antigen cross reacting with MMTV indicates that human material will be available that will accelerate studies on the involvement of viruses in the cause of breast cancer. The chemotherapy studies have already been of value to breast cancer patients in the United States and the results of these studies are encouraging American chemotherapists to treat patients with rapidly progressing breast cancer who in the past had not been tried on chemotherapy. The immunologic studies demonstrate the integrity of the immune system in patients with RPBC, providing guidelines to management of such patients.

Proposed Course:

Project A

Serologic techniques will continue to be applied to populations of normal individuals at high and low risk to NPC. Additional longitudinal studies on patients with BL and NPC will be performed to increase our understanding of which tests will be of value in the monitoring of patients with these tumors. Continued attention will be given to the development of a primate model for BL. All available serologic and CMI assays will be applied to determine which immunologic components correlate with susceptibility and resistance to EBV-associated tumors in nonhuman primates. A number of drugs and biologic modifiers will then be screened by the in vitro assay for activity against EBV and these materials will then be applied to in vivo studies of primates infected with EBV. Some of the materials with anti-EBV activity already in clinical use will be evaluated for therapeutic trials in patients with BL.

Project B

Additional patients will be studied by the immunoperoxidase assay and other virologic techniques to determine the nature of the antigen cross-reacting with MMTV in Tunisian patients with breast cancer. The monitoring of patients under chemotherapy will be continued with an attempt to identify parameters associated with a favorable response to chemotherapy. Immunologic studies with more defined antigens will be continued to define similarities and differences between breast cancer patients in the U.S. and Tunisia.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04825-07-LVC

PERIOD COVERED
September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)
Physico-chemical Studies of Viral Nucleic Acids and Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. D. K. Haapala Microbiologist LVC, NCI

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Viral Carcinogenesis

SECTION Virus Control Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Last year we reported that the endogenous cat virus, RD-114, was able to productively infect 13 of 13 cat embryonic cell lines tested. Studies with additional embryos have served to extend this observation.

Further biological studies have revealed the presence of two distinct sub-types of RD-114 virus. Additionally, cat embryo cells can be characterized as "permissive" or "restrictive" based on results obtained with the two virus sub-types.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To characterize and utilize viral nucleic acids and proteins as diagnostic and analytical tools for probing the nature and mechanism of viral oncogenesis.

To study different virus-cell interactions to determine the nature of the operant control mechanism(s).

Methods Employed:

Standard biochemical, biophysical, cell and virus culture methods are used. These include ultracentrifugal, chromatographic, enzymological, nucleic acid hybridization, gel filtation, electrophoretic, immunological and *in vitro* protein synthesis techniques.

Major Findings:

1. We have employed the RD-114 pseudotypes of Moloney sarcoma virus (MSV) in a screening method for cat embryo cell sensitivity to endogenous virus. Results of this method showed that certain embryos were restrictive, others permissive, based on whether or not MSV focus formation occurred.

2. RD-114 virus was purified on the two cell types by numerous terminal dilutions. When plated back on the two cell types, results were consistent with the isolation of two distinct RD-114 sub-types.

3. A "permissive" embryonic line has been used to generate an S+L- line nonproductively transformed by MSV. This line serves as a rapid and sensitive indicator for both RD-114 sub-types.

Significance to Biomedical Research and the Program of the Institute:

The use of viral proteins and nucleic acids as markers in the study of oncogenesis is a basic essential. Increasing the number and availability of such markers has obvious utility in increasing our ability to understand the oncogenic process.

The question of how a cell organizes vast amounts of genetic material has been and is of fundamental importance. Cancer causing viruses provide one of several tools which can be used to examine the control of a few specific gene products and so become of general interest. In addition, these studies may prove useful in determining how to control certain cancers.

Proposed Course:

Studies on the control of RD-114 expression in cat cells will be continued. Moloney MSV will be included in these studies to provide the additional capabilities of detecting both early and late viral expression in a clean genetic background.

Other cat embryo cells and RD-114 virus isolates will be studied to determine if more than two sub-types exist. Additionally, biochemical methods will be employed to further characterize and distinguish the two sub-types isolated to date.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04826-08-LVC

PERIOD COVERED
September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)

Expression and Control of Transformation by Murine Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. P. J. Fischinger,	Head, Virus Control Section	LVC, NCI
D. G. Blair,	Expert Consultant	LVC, NCI
S. Nomura,	Microbiologist	LVC, NCI
D. K. Haapala,	Microbiologist	LVC, NCI
W. G. Robey,	Research Chemist	LMV, NCI
G. F. Vande Woude	Head, Tumor Virus Biochemistry Section	LMV, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Virus Control Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention
NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	0.5	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cloned MSV proviral DNA and various pieces containing src were transforming in transfection assays. src and one extended terminal redundancy represented the minimal active piece.

Tumors were induced by MSV in Japanese quail. These regressed with high frequency and such quail sera were examined for antiviral and anti nonstructural protein reactivities.

Reversion from MSV transformed state was studied at the proviral DNA and RNA levels in mouse and heterologous species cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To identify transforming sequences of cloned MSV proviral DNA in cell culture systems. To develop MSV regressor systmes in genetically unrelated hosts to study putative anti src antibodies. To attempt in cell culture studies to identify the nature and mechanisms of reversion from the transformed state.

Methods Employed:

The usual method of tissue culture including propagation and derivation of normal and tumor cultures. Standard virological assays such as focus assay for both transforming and nontransforming oncornaviruses, soft agar assays, isotopic precursor incorporation, isolation and purification of viruses, density gradient centrifugation. Isolation of protein and nucleic acid components of viruses and cells including reverse transcriptase assays, nucleic acid hybridization, restriction enzyme analyses, and cloning of DNA fragments.

Immunological and biochemical procedures in isolation and induction of immunoglobulins and the purification of various natural host protective factors. Protein and lipoprotein analyses, radioimmune precipitation, immunofluorescent analysis of in vivo and cultured cell samples. Sero-immunoprophylaxis protocols in several in vivo model systems.

Major Findings:

We devised cloned mink cell systems transformed by two molecularly different isolates of MSV, prepared specific complementary DNA probes to common and src portions of MSV to identify MSV integration sites and in collaborative studies (G. Vande Woude) isolated the MSV proviral DNA in the integrated form in mink cells containing only one MSV DNA per haploid genome. The method of recombinant DNA cloning in lambda bacteriophage (λgtWES λB) was chosen. Two isolates of Moloney MSV were chosen: one was the m1 MSV known to be smaller and which translated a gag precursor p60 polyprotein, and the HT-1 MSV which was rescued from hamster tumors. This HT-1 MSV was longer, contained as much or more gag gene information as determined by hybridization but did not translate any gag product in any cell system. Direct heteroduplex comparisons showed graphically the homology of the subsituted src region as well as the intrinsic differences and deletions in the two genomes and directly showed that terminal redundant sequences (TRS) were present at both ends of MSV DNA and that the host flanking sequences were completely different for each MSV. The proviral sequences with mink flanks in lambda (λ) were 12.3 kbp and 7.0 kbp fragments of HT-1 and m1 respectively. The two MSV's were co-linear and the 1.5 kbp difference within the genome was due to two 0.8 kbp deleted regions in m1 MSV. An interesting and useful feature was that one of the TRS sequences was retained in λ during propagation in a rec$^+$ E.

coli host. Our major direction was to identify the biological activity of cloned MSV utilizing cloned whole genomes of the MSV isolates (m1 and HT1), as well as specific subgenomic fragments of these two genomes cloned in the plasmid pBR322. The biological activity of specific DNA fragments derived from thse well characterized clones was measured in DNA transfection assays utilizing established procedures. This involved precipitating the DNA's, in the presence of sheared calf thymus carrier DNA, with calcium and phophate, and adding this DNA precipitate to uninfected cells. W h o l e cloned proviral DNA transformed cells in culture with a high efficiency ($\sim$5 x 10^4 foci/picomole DNA). Both indirect immunofluorescence and direct immunoprecipitation indicated that cells transformed by the m1 MSV proviral DNA were able to translate the MSV coded p60 precursor polyprotein. The MSV genome present in these cells was also rescuable following superinfection with replicating helper leukemia viruses in most of the single focus isolates tested. Furthermore, infection of transfected cells with optimal amounts (m.o.i. 0.1) of helper MuLV resulted in the appearance of early, helper dependent foci approximately 5-7 days later, while in the absence of helper, foci did not appear until 10-15 days after transfection.

Subgenomic, src containing fragments from either the 3' or 5' portion of the MSV genome also transformed efficiently, although the specific infectivity was reduced 6-fold. The 3' fragament pm13 was not rescuable, but a small amount of infectious transforming virus could be rescued from pHT25 transfected cells following MLV superinfection. While these fragments transformed with relatively high efficiency, the internal fragments pHT10 and pHT11 transformed with low efficiencies. These represent the minimal transforming fragments of MSV identified at this time. They consist of 1.2kb of src together with 1.0 kb of MuLV sequences immediately adjacent to the 5' end of src.

It is apparent that while the sequences present in pHT10 or pHT11 might be sufficient for transformation, the presence of one copy of the 0.6 kb TRS from either the 5' of the 3' end of the genome increased the efficiency of cell transformation about 1000-fold. Further support comes from experiments using pHT10 fragment joined in vitro to the non-transforming fragment pHT13. The resulting clones (pHT21 and pHT22) transformed 1000 fold more efficiently than pHT10. Furtherfmore, a mixure of pHT10 and pm1sp, a clone which contains a single copy of the TRS and the associated mink flanking sequences, transformed 300-fold more efficiently than pHT10 alone.

After several attempts in various species we found (with W. G. Robey) that MSV as MSV(B-MuX) pseudotype could give rise to tumors in quails with high efficiency ($\sim$40%) if quails were inoculated with quail secondary cells infected for two days with MSV(B-MuX). Control infections with B-MuX or B-MuX infected cells yielded no tumors. The tumors were predominantly fibrosarcomas although several lymphosarcomas were identified. Tumors were either progressors, steady state, or regressors. A remarkable finding was that MSV replicated in quail to highest titers ever observed and that

all tumors were potential producer tumors. Cell lines which produced virus were established from MSV tumors in long term cell cultures and atypical processing of MSV proteins was observed. Pools of sera prepared from birds with regressing or persisting tumors had high titers of antibody. The main response was specifically directed against the B-MuX gp70 component as identifiable by immune precipitation or neutralization. No anti MSV p60 nor any antibody against phosphokinase phosphoprotein structure were ever found. Residual antibodies are being scrutinized but it does not appear that the putative product of the MSV src is analogous to the avian sarcoma virus coded phosphokinase.

Reversion from the MSV transformed state to a state of normalcy in culture was found to occur with a potentially high frequency. In mouse cell revertants, the MSV genome persisted in the DNA but could not be rescued by helper virus superinfection. Multiply cloned revertant cells had most properties of normal cells except for lectin agglutinatibility. Rarely some cloned revertants back-transformed and from a fraction of these MSV could be rescued. Some mouse cell revertants demonstrated that a differential transcriptional control exists over the 5' and the 3' regions of the MSV genome, because in some revertants RNA specific for the shared regions of MSV was present whereas the RNAs for the MSV specific regions was absent.

A rather different mechanism was found in revertants isolated from MSV transformed cat cells. In all cases no MSV rescue was seen and no MSV associated products or properties were observed. Molecular analysis with total MSV cDNA or cDNA specific for common or src sequences revealed that MSV had been evicted. Concommitant analysis of feline leukemia virus, or feline xenotropic virus-like sequences showed that these were not obviously altered during the reversion process.

Significance to Biomedical Research and the Program of the Institute:

Isolated presumptive tumor virus genes cloned in bacteria, and proven to be functional biologically is the obvious approach at dissecting and finally sequencing this structure which should lead to an exact identification and potential translation of the critcial interactive regions. The development of novel transforming systems where transforming sequences and host DNA have no detectable homology should elucidate host control mechanisms. Reversion process in defined systems relate directly to cellular mechanisms of control.

Proposed Course:

Further analysis of permutations of cloned DNA's of the MSV src, TRS, and MuLV derived regions will unequivocally establish those nucleotide sequences critical to transformation. Ongoing parallel investigations of the above will sequence critical DNA sites relative to promoter and operator activities. Cells transformed with a single TRS-src derivative

will be examined as well as the nature and content of flanking cellular sequences. MSV avian regressor tumor systems will be examined further specifically with the potential of generating nonproductive tumors in quail and the identification of all reactive antibody populations relative to MSV viral and MSV transformed cell products. Attempts at understanding MSV processing changes will be followed as well as potential inductions of tumors with ts MSV.

Reversion will be studied in mouse revertant cells with the examination of messenger RNA's using Northern blot transfer technology to see whether cellular sarc or endogenous MuLV genes play a role in the control of reversion.

Publications:

Vande Woude, G. F., Oskarsson, M., McClements, W. L., Enquist, L. W., Blair, D. G., Fischinger,, P. J., Maizel, J. V., and Sullivan, M.: Characterization of integrated Moloney sarcoma proviruses and flanking host sequences cloned in bacteriophage lambda. In: The Transformed Cell, Cold Spring Harbor Symp. Quant. Biol. Vol. 44. In Press, 1979.

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Fischinger, P. J., Blevins, C. S., Frankel, A. E., Tuttle-Fuller, N., Haapala, D. K., Nomura, S., and Robey, W. G.: Reversion of cat cells transformed by MSV: Biological, immunological and molecular properties of flat variant cells. Cancer Res. Submitted June, 1980.

Robey, W. G., Kuenzel, W., Vande Woude, G. F., and Fischinger, P. J.: Induction of tumors by Moloney murine sarcoma virus in Japanese quail and charcterization of tumor cell lines in culture. J. Natl. Cancer Inst. Submitted June, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04829-06-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Endogenous Retroviruses of feral Rodents: Genetic Variation and Tumorigenicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Callahan	Microbiologist	LVC	NCI
OTHER:	G. J. Todaro	Medical Officer	LVC	NCI
	J. Schlom	Microbiologist	LVC	NCI
	U. R. Rapp	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Colonies of Asian species of Mus established in this Laboratory were used as a basis for the study of genetic variability of murine endogenous retroviruses and the genetic elements which affect their expression and/or involvement in neoplastic disease. Four different classes of endogenous retroviruses have been isolated from Asian Mus species. These include two distinct classes of type-C virus (designated C-I and C-II), a novel class of viruses (M432 group) and milkborne type-B viruses. The type-C and M432 group of viruses were obtained either from cultured cell lines which spontaneously began producing virions or by induction of tissue culture cells with pyrimidine analogues followed by cocultivation with sensitive indicator cell lines. The major internal viral protein (p30) of the C-I class competes in a woolly monkey - gibbon ape group-specific radioimmunoassay, whereas, the respective protein of the C-II class shows no competition.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To use the colonies of Asian species of Mus established in this Laboratory as a basis for the study of genetic variability of murine endogenous retroviruses and the genetic elements which affect their expression and/or involvement in neoplastic disease. Specifically, (1) isolate and characterize endogenous retroviruses from the different Asian species of Mus and compare them, at a molecular level, with known endogenous retroviruses of M. musculus and infectious primate type-C viruses; (2) determine whether new viral isolates are capable of inducing neoplastic disease and/or whether their expression is associated with different forms of neoplasia in their nature host; (3) compare the relative evolutionary stability of endogenous retroviral genes, their arrangement in cellular DNA, and the nature of adjacent cellular sequences.

Methods Employed:

Outbred colonies of Asian species of mice were established in 1976-77 from mice trapped in Thailand by Dr. J. Marshall (Smithsonian Institute). The impetus for starting the colonies was two fold. First, studies in our laboratory on the origin of the woolly monkey - gibbon ape group of infectious primate type-C viruses suggested that these retroviruses were of murine origin (Benveniste and Todaro, Proc. Natl. Acad. Sci.(USA) 70:3316, 1973; Sherr et al., J. Virol. 15:1440, 1975). Since certain natural populations of gibbon apes in Southeast Asia are infected with this class of infectious retroviruses, murine species of Thailand seemed a logical place to begin looking for the progenitor of the infectious virus. Second, earlier studies with endogenous type-C retroviruses isolated from feral and inbred strains of M. musculus indicated that at two different biological levels (1) the individual mouse cellular genome, and (2) comparison of one mouse (or inbred strain) genome with those of other mice; there exist groups of related but not identical endogenous type-C virogenes (Callahan et al., J. Virol. 14: 1294, 1974; Callahan et al., J. Virol. 15:1378, 1975). We chose to enlarge the scope of the study of genetic variation among endogenous murine retrovirogenes and the genetic elements which regulate their expression by surveying other species of the genus Mus.

Major Findings:

Four different classes of endogenous retroviruses have been isolated from Asian Mus species. These include two distinct classes of type-C virus (designated C-I and C-II), a novel class of viruses (M432 group) and milk-borne type-B viruses. The type-C and M432 group of viruses were obtained either from cultured cell lines which spontaneously began producing virions

or by induction of tissue culture cells with pyrimidine analogues followed by cocultivation with sensitive indicator cell lines.

A. Endogenous Type-C Viruses.

Of the type-C viruses isolated from the different Asian species two subclasses (C-I and C-II) could be distinguished by the following criteria:
(a) Host Range: C-I viruses cannot productively infect tissue culture cells of murine origin, but do grow on tissue culture cells derived from other mammalian species, whereas, the host range of C-II viruses include murine tissue culture cells.

(b) The major internal viral protein (p30) of the C-I class competes in a woolly monkey - gibbon ape group-specific radioimmunoassay, whereas, the respective protein of the C-II class shows no competition. Reciprocal results are obtained in a M. musculus MuLV group-specific radioimmunoassay. While the p30 proteins of C-I and C-II viruses can be distinguished by their unique antigenic determinants, they also share other determinants in common. Thus in an interspecies radioimmunoassay, which detects antigenic determinants common to endogenous porcine and infectious primate type-C viruses, C-I and C-II, both compete, whereas, rat and hamster type-C viruses do not compete in this assay.

(c) Classification of type-C viruses into C-I and C-II classes can also be performed using antisera directed toward viral reverse transcriptase. Using an antiserum prepared to the polymerase of SSAV, the activities of polymerases of C-I viruses are inhibited to a greater extent than the enzyme of C-II viruses. Conversely, antisera to the polymerases of standard MuLVs preferentially inhibit the reverse transcriptases of C-II viruses, but cross react to a lesser extent with enzymes from C-I viruses.

(d) Sequences related to the C-I viral genome are highly conserved in the cellular DNA of all murine species and other rodent genera (comparable to the conservation of nonrepetitive cellular DNA). In contrast, the C-II viral genome is poorly conserved, even between members of the same species. Consistent with this observation, the genomes of C-I and C-II viruses share only partial nucleic acid sequence homology as measured using S1 nuclease.

Type-C-I retroviral isolates have been obtained from each of the Asian species of Mus, except M. musculus and from the rodent Vandeleuria oleracea. In M. musculus, although C-I viral related sequences are present in the cellular DNA, they may be present in a cryptic form or their expression may be more tightly regulated than in other rodent species. We are currently pursuing the former possibility by comparing the arrangement of C-I related virogene sequences in M. musculus with those in M. cervicolor by restriction enzyme mapping. In comparison to C-I viruses, C-II viral isolates have been obtained only from M. cervicolor and M. cookii, in addition to M. musculus. The possibility that not all individuals of a given species, in fact,

contain C-II endogenous virogenes is currently being investigated. Such a situation has been reported for type-B virogenes in certain populations of M. musculus. In other work, we are investigating the relationship between C-I and C-II viral genomes. The properties of the two classes of retroviruses (relative conservation of genomic sequences in cellular DNA, the partial homology between viral genomes and common antigenic determinants on viral proteins) suggests that the C-I class of endogenous retroviruses may be the evolutionary progenitor of the C-II retroviruses.

Since C-I retroviruses are readily isolated from cultured cells and are expressed in a number of spontaneous tumors of Asian mice, we are currently testing their oncogenic potential in other mammalian species. Two C-II viral isolates obtained from leukemic M. cervicolor mice do induce this disease upon inoculation into NIH Swiss or M. cervicolor mice. In collaboration with Dr. U. Rapp (Z01-CP-04868-05-LVC), we are attempting to determine whether there are specific integration sites for the M. cervicolor C-II retroviruses, which correlate with the development of the disease in infected NIH Swiss mice. In these experiments we will take advantage of the low level of nucleic acid sequence homology between the M. cervicolor viral genome and uninfected M. musculus cellular DNA. Using restriction enzyme mapping, we will determine whether there are specific patterns of cellular DNA containing viral related sequences which correlate with the development of the tumor.

B. The M432 Group of Endogenous Retroviruses.

The M432 virus was isolated by the cocultivation of a M. cervicolor spleen with NIH Swiss cultured cells. Cells releasing the virus contain intracytoplasmic-A-particles. The mature viral particles had, by electron microscopy, a novel morphology, distinct from known types-B, -C and -D retroviruses. Subsequently, two additional viral isolates were obtained from M. cervicolor popaeus and M. caroli. The host range of these viruses is restricted to cell cultures derived from M. musculus. The M432 virus, like other members of Retroviridae, contains an RNA genome which is approximately 35s in size. DNA sequences related to the M432 viral genomes are reiterated 25 times per haploid genome in M. cervicolor and M. caroli cellular DNA. Viral related DNA sequences can also be detected in other species of this genus including M. dunnei, M. cookii, M. musculus and to a lesser extent in M. pahari and M. shortridgei. The viral associated proteins include a Mg^{++} ion dependent reverse transcriptase (70,000 daltons), 2 glycosylated envelope proteins (gp69 and gp33), a major internal protein (p24), a second internal protein (p16), and phosphoprotein (p12). Using antisera against the major internal protein (p24), we developed a competition radioimmunoassay for this protein. Protein extracts of the M432 viral isolates from M. cervicolor and M.caroli compete, whereas, extracts of type-B, type-C and type-D retroviruses do not compete in this assay. Consistent with this observation is the absence of nucleic acid sequence homology between M432 viral genomes and the genomes of other Retroviridae.

The M432 virus has been tested in BALB and NIH Swiss mice for its oncogenic potential. Although expression of viral proteins in infected animals has been detected, we have no evidence that the virus is a causative agent for neoplasia. In other studies, we have tested various spontaneous tumors which have appeared in the M. cervicolor colony as well as normal tissues in the p24 radioimmunoassay and found no evidence for viral expression. In contrast, low levels of viral RNA expression could be detected in various tissues of normal M. cervicolor and M. musculus. Moreover, many tissue culture cell lines derived from M. musculus and transformed with a variety of agents (viral and chemical carcinogens) express detectable levels of M432 related viral RNA.

Recently, have shown that the RNA genome of intracisternal-A-particles (IAP) of M. musculus share nucleic acid sequence homology with the M432 viral genome. In collaboration with Dr. Kuff, we have found that the major IAP core protein (p73) is immunologically related to the major internal protein (p24) of the M432 virus. Antisera prepared against the IAP p73 reacts only with the M432 p24 protein and not with other viral associated proteins. Whether the IAP genome of M. musculus represents a defective variant of the M432 viral genome or is a hybrid between M432 related sequences and other cellular sequences is unclear. We are currently cloning the M432 viral genome in bacteriophage lambda, in order to define the relationship between the viral and IAP genomes.

IAPs have been observed in large numbers in certain tumors of M. musculus, including mineral oil induced plasma cell tumors, certain teratocarcinoma tissue culture cell lines and certain BALB/c mammary hyperplastic nodule lines. In normal M. musculus tissues, large numbers of IAP have only been observed during early stages of embryonic development (2-8 cell stage). Recently, in collaboration with Dr. P. Calarco, we have also observed these particles in similar stages of embryonic development in M. cervicolor and M. pahari. Studies are planned to determine which M432 viral proteins are being expressed during embryonic development.

C. Genetic Complexity and Organization of Endogenous Retroviral Genes.

In many mammalian species, the genomes of endogenous retroviruses are represented in cellular DNA in multiple copies. Using labeled viral DNA, the reiteration frequency of viral-related sequences for each class of M. cervicolor viruses was estimated. Both the type-C-I and C-II virogenes are reiterated approximately six times per haploid cellular genome, while M432 virogenes are reiterated approximately 25 times. Recently, Morris et al. (J. Mol. Biol. 114:73, 1979), have estimated that there are at least 25 copies of type-B virogenes (MMTV) her haploid genome in M. cervicolor.

The approximate genetic complexity of a single viral genome is 10,000 base pairs and that of the cellular DNA is 1.5×10^9 base pairs/haploid genome. Thus, at least 0.04% of the M. cervicolor cellular genome contains genetic

information related to that of endogenous retroviruses; a value comparable to the genetic complexity of the structural gene coding for 28s and 18s ribosomal RNAs (Graubatz and Cutler, Biochemistry 14:760, 1978). These represent minimal estimates since additional subclasses of retroviruses may yet be isolated from one or more of these Mus species. Similar calculations can be made for M. musculus. In this species, sequences related to intracisternal A particles are present in approximately 500 to 1,000 copies per haploid genome and therefore, alone represent 0.3 to 0.6% of the cellular DNA. A similar, although not as dramatic, disparity in reiteration frequency of C-I viral related sequences is observed in members of the genera Mus and Vandeleurea oleracea. In the latter genus, these sequences are reiterated 75-100 times per haploid genome.

Currently studies are being initiated to determine the arrangement of endogenous retrovirogenes in the cellular DNA of M. cervicolor and the nature of the adjacent cellular sequences. Toward this goal, cellular DNA fragments containing viral related sequences are being cloned with bacteriophage lambda. In preliminary work, we have determined the pattern of M432 related DNA fragments from different murine species using restriction enzymes and the Southern blotting technique. The results indicate that although there appears to be diversity between individual copies of viral sequences in the cellular DNA of an individual mouse, this pattern is conserved among different individuals of the same species, whereas, major differences are observed when comparing members of different murine species. These results are in marked contrast to the patterns observed by others for M. musculus cellular DNA sequences related to type-B and C endogenous retroviruses (Cohen and Varmus, Nature 278:418, 1979; Steffin and Weinberg, Cell 15:1003, 1978).

Significance to Biomedical Research and the Program of the Institute:

The extensive genetic and immunological data available for inbred and feral populations of M. musculus have made it one of the primary model systems for the study of viral and chemical carcinogenesis. However, by the restriction of this work to selected inbred strains or feral populations only a portion of the total genetic variation in the species or genus pool can be studied.

Inbred and feral populations of M. musculus release two classes of endogenous retroviruses (type-B and type-C). The study of the Asian species of Mus has led to the identification of at least two additional previously unrecognized classes of endogenous retroviruses. One of these designated M432, is novel in the sense that it is unrelated by a variety of criteria to all other classes of retroviruses, but is related to the intracisternal A particles observed in some tumors and early embryo's of M. musculus. The second class (type-C-I) is related to the woolly-gibbon ape group of infectious primate type-C viruses which are horizontally transmitted in primates. The broad host range of type-C-I viruses suggests that other examples of interspecies transmission of this class of endogenous rodent type-C virus may be encountered. Moreover, the conserved nature of the type-C-I viral genome in

the cellular DNA of rodents and the partial sequence homology between C-I and C-II viral genomes is consistent with the possibility that C-I retrovirogenes are the progenitors of the type-C-II class of retroviruses. It is this class of viruses which is associated with neoplastic disease in mice.

As a result of the identification of these new classes of endogenous retroviruses and the fact that they are all present in the cellular DNA in multiple copies, it is apparent that they represent a significant fraction of the cellular DNA. The total compliment of endogenous retroviral sequences accounts for a minimum of 0.04% to 0.3% of the Mus cellular genome. This calculation takes into consideration only the genetic information corresponding to viral associated structural proteins. Additional genetic information would be required to account for the various mechanisms the species has evolved to regulate the expression of these virogenes. Continued work with these colonies offers the possibility of identifying as yet new genetic elements involved in neoplasia, as well as the expression of retroviral genes. Moreover, the presence of multiple copies of virogenes in the cellular DNA presents a unique opportunity to study the effect of genetic variation on the organization of cellular DNA using as a probe the retroviral genomes.

Proposed Course:

This program of research will be continued and extended to further characterize the viruses isolated and to define their role in the induction of malignancy.

Publications:

Callahan, R., Meade, C., and Todaro, G. J.: Isolation of an endogenous type-C virus related to the infectious primate type-C viruses from the Asian rodent Vandeleuria olracea. J. Virol. 30:124-131, 1979.

Kuff, E. L., Callahan, R., and Howk, R. S.: Immunological relationship between the structural proteins of intracisternal-A-particles of Mus musculus and the M432 retrovirus of Mus cervicolor. J. Virol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04840-10-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies on the Etiology and Control of Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.A. Leiseca	Research Biologist	LVC NCI
Other:	P.H. Levine	Medical Director	LVC NCI
	K.S. Sundar	Fogarty Fellow	LVC NCI
	A. Hellman	Assistant Chief	LCMB NCI

COOPERATING UNITS (if any)
Dr. David Martin, Litton Bionetics, Kensington, Maryland

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Clinical Studies Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project seeks to study the pathogenesis of virus-associated tumors in man, particularly lymphoma, with an attempt to utilize animal models whenever possible. The studies are designed to provide information as to the possible viral etiology and means of controlling the tumors under evaluation.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To evaluate non-human primates as models for studies on the etiology and control of human cancer.

Methods Employed:

Six white-lipped marmosets (WLM) and 15 mice were inoculated with infective B-95 Epstein-Barr virus. The results of a study on the inoculation of chimpanzees, rhesus, and cynomolgus monkeys with EBV were correlated and the results of antibody patterns in animals inoculated with EBV were compared with those of controls. Thoracic duct cannulation was used as a means of immunosuppressing rhesus monkeys prior to inoculation with the B-95 transforming strain of EBV. Animals were followed clinically and blood samples were obtained for immunologic studies. Thoracic duct lymphocytes were collected for infectivity studies with an attempt to infect rhesus monkey lymphocytes with various lymphotropic oncogenic herpes viruses.

Major Findings:

Of six WLM inoculated with EBV, one developed a malignant lymphoma but no disease or antibody production was observed in the inoculated mice. Persistent antibody to EBV indicated that 14 of the inoculated animals were infected with EBV. Despite heavy immunosuppression and immunostimulation in some of them, no disease appeared to result from EBV inoculation. Thoracic duct cannulation continued to be a safe and effective means of depleting T-lymphocytes in rhesus monkeys.

Significance to Biomedical Research and the Program of the Institute:

The development of a useful animal model for EBV infection in man continues to have a high priority for the program. The relative availability of rhesus monkeys and their phylogenetic proximity to humans make this animal a potentially valuable model for EBV infection in man. The production of lymphoma in a WLM is important because of the relative availability of this animal for experimental studies.

Proposed Course:

Primates already inoculated with EBV will continue to be monitored for the appearance of lymphoma. Attempts will be made to find other forms of tumor induction in rhesus monkeys and additional experimental models will be evaluated.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04842-09-LVC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Biochemistry and Biophysics of Leukemia and Sarcoma Virus Nucleic Acids and Polypeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L.A. Phillips	Research Microbiologist	LVC	NCI
OTHER:	M.S. Kang	Visiting Fellow	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Office of the Chief, LVC

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on the biochemical and biophysical properties of nucleic acids and poly peptides of mammalian leukemia and sarcoma retroviruses have shown that: (1) The subunit RNA of sarcoma viruses examined is smaller then the subunit RNA of leukemia viruses. (2) Homopolyribonucleotide sequences (A,C,G) have been detected in the RNA of retroviruses from avian to primate isolates. (3) Poly(C) and poly(G) sequences, present in retrovirus RNA are transcribed into poly(dG) and poly(dC) sequences, respectively, in the retrovirus DNA. (4) The 3'-terminal poly(A) sequences of retrovirus RNA are not transcribed into poly(dT) sequences, in retrovirus cDNA. (5) Little or no poly(C) and poly(G) sequences have been detected in the nucleic acids examined from nonmalignant mammalian and human embryonic cells examined. (6) Genomic and subunit RNA of mammalian retroviruses have intrastrand and interstrand regions of double-stranded RNA.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To establish a sound scientific basis for a molecular approach to cancer prevention and treatment for those human cancers in which retrovirus information has been detected. To look for and to characterize, if detected, unique specific nucleotide sequences present in retrovirus nucleic acids which may either be absent or present in insignificant amounts in cellular nucleic acids from nonmalignant mammalian and human cells.

Methods Employed:

For characterization of the viral nucleic acids and polypeptides from retroviruses, standard biochemical and biophysical techniques (analytical and preparative ultracentrifugation, chromatography, nucleic acid hybridization, isotopic labeling, polyacrylamide gel electrophoresis, peptide and oligonucleotide fingerprinting, electrofucusing, in vitro protein synthesis, enzymology, etc.) are being employed. Improved tissue culture techniques are being employed for the growth of retroviruses, infectivity assays of retroviruses. Immunodiffusion, radioimmune analysis, molecular cloning, agarose gel electrophoresis, southern blot transfer analysis, viral RNA and DNA sequencing, and restriction endonuclease mapping are being used. Amino acid and glycoprotein analysis are to be used.

Major Findings:

Results from the biochemistry and biophysics of retrovirus and cellular nucleic acids studies are: (1) The subunit RNA of mammalian sarcoma viruses is approximately 5.0 to 5.8 kilobases in contrast to the 5.8 to 10.5 for the subunit RNA of mammalian leukemia viruses. (2) The 3'-terminus of some viral RNA subunits has poly(A) sequences, or tracts rich in AMP, of variable chainlengths of 300 nucleotides of less. (3) Poly(A), poly(C), and poly(G) sequences, or tracts rich in AMP, CMP, and GMP respectively, are present in retrovirus RNA from avian to primate isolates. However, poly(U) sequences, or tracts rich in UMP, are absent in retrovirus RNA. (4) Four classes of mammalian viral RNA subunits have been identified according to their homopolyribonucleotide tract composition and the four subunits assembled into a proposed genomic model for mammalian retroviruses. (5) The poly(C) and poly(G) sequences in the viral RNA are transcribed into poly(dG) sequences, or tracts rich in dGMP, and poly(dC) sequences, or tracts rich in dCMP, in the viral complementary DNA (cDNA). However, the 3'-terminal poly(A) sequences of retrovirus RNA are not transcribed into poly(dT) sequences, or tracts rich in TMP, in the viral cDNA. (6) Comparatively little or no poly(dC) and poly(dG) sequences have been detected in the DNA examined from normal mammalian and human

cells but these sequences are present in the DNA of retrovirus infected and transformed cells, as well as chemically transformed cells, of mammalian and human origins. (7) Genomic and subunit RNA of mammalian leukemia and sarcoma viruses have regions of double-strandedness rich in CMP and GMP.

Significance to Biomedical Research and the Program of the Institute:

A molecular approach to cancer prevention and treatment for human cancers in which retrovirus information has been detected, most noticeable of these are breast cancer and leukemia, has been justified, in part, from our biochemical and biophysical studies on the structure and nucleotide composition of retrovirus and cellular nucleic acids. Analysis of homopolyribonucleotide sequences in retrovirus RNA revealed the existence of 3'-terminal poly(A) sequences and internal poly(C) and poly(G) sequences. These sequences have been detected in the RNA of retroviruses from avian to primate isolates. Poly(A) sequences have also been found in all cellular messenger RNA(mRNA), except for histone mRNA, by many investigators. We have shown that comparatively little or no poly(C) and poly(G) sequences exist in poly(A) positive mRNA examined from nonmalignant and embryonic cells. The poly(C) and poly(G) sequences present in viral RNA are transcribed into poly(dG) and poly(dC) sequences respectively in the viral cDNA, before it becomes integrated into cellular DNA where it apparently remains in an inactive, repressed state until it is activated or derepressed either spontaneously or by chemical or physical carcinogens. The absence of significant amounts of these homopolydeoxyribonucleotide sequences in the cellular DNA from nonmalignant human embryonic cells and their presence in DNA from retrovirus infected and transformed cells, as well as the DNA from chemically transformed cells, suggest an experimental basis for a possible molecular approach to cancer prevention and treatment for cancer in which retrovirus information has been detected. Perhaps, the transcription of the integrated viral genes with their unique sequences of dCMP and dGMP could be selectively blocked by either in situ hybridization with complementary synthetic homopolymers to the poly(dC) and poly(dG) sequences in the integrated retrovirus DNA or specifically blocked by sequence specific drugs or chemically modified antibiotics binding or intercalating between the viral specific sequences of dCMP and dGMP. Although human cancers in which retrovirus information has been detected are most probably multifactorial, this unique approach seems worthy of exploring for the prevention primarily and the treatment of cancer.

Proposed course:

These essential biochemical and biophysical studies on the nucleic acids of mammalian retroviruses and mammalian and human cells will be continued. They will be done in order of their complexity and available technology, as well as the availability of adequate professional and technical assistance and funds. Our major research efforts will continue to be directed toward the establishment of a sound scientific basis for a molecular approach to

cancer prevention and treatment. The following biochemical and biophysical studies will be performed on nucleic acids of retroviruses, cells, and viral glycoproteins.

RNA studies: (1) Determination of the location(s) of the poly(C) and poly(G) sequences in the retrovirus RNA by controlled PNPase phosphorolysis of poly-(A) positive viral RNA subunits from their 3'-termini, by heteroduplex analysis when it becomes sensitive enough to resolve structures with less than 40 base pairs. (2) Demonstration of the poly(C) and poly(G) sequences in the viral RNA by two-dimensional chromatography in agarose of RNase T1 and RNase C excised tracts. Presently, the like sequences of CMP and GMP in retrovirus RNA have been detected by polynucleotide agarose affinity chromatography, by hybridization or annealing studies, by specific chemical binding studies, and by the ability of oligo(dC) and oligo(dG) to serve as primers in the in vitro synthesis of viral cDNA. (3) Determination of the base composition of excised dsRNA fragments from retrovirus RNA. (4)Determination as to whether the p12 viral polypeptide binds to single-stranded or the double-stranded portion of genomic and subunit viral RNA. (5)Reassociation of the four types of subunits into genomic viral RNA in vitro. (6) Characterization of the interstrand and intrastrand double-stranded regions in the genomic and subunit RNA of mammalian retroviruses as to their nucleotide composition, their definitive chain-lengths, and their possible functions. (7) Determination of the role(s) of the poly(A), poly(C), and poly(G) sequences in genomic and subunit viral RNA. (8) Continued examination of the poly(A) positive mRNA from nonmalignant cells, from retrovirus infected and transformed cells, and chemically transformed cells of human origin for the absence or presence of poly(C) and poly(G) sequences.

Planned DNA studies: (1) Location of the poly(dC) and poly(dG) sequences in subunit-length cDNA copies of retrovirus RNA. (2) Continued examination of the DNA from nonmalignant cells, from retrovirus infected and transformed cells, and from chemically transformed cells of human origin for the absence or presence of the poly (dC) and poly(dG) sequences. (3) Determination of the kilobase length of the cDNA transcripts when oligo(dA), oligo(dC), and oligo(dG) are used as primers in the in vitro synthesis of cDNA.

Planned glycoprotein studies: Determination of the effect(s) of known glycoprotein inhibitors on retrovirus assembly and replication.

Publications:

Park, J.J., Phillips L.A.: Isolation and purification of double-stranded RNA fragments from retrovirus RNA. Prep. Biochem. 9: 261-272 (1979).

Elbein, A.D., Fafford, J. and Kang, M.S.: Inhibition of Lipid-Linked saccharide synthesis, comparison of Tunicamycin, Streptovirudin and Antibiotic 24010. Arch. Biochem. Biophys. 196, 311-318 (1979).

Kang, M.S. and Elbein, A.D.: Incorporation of Glucose into Lipid-Linked Saccharides in Aorta and its Inhibition by Amphomycin. Arch. Biochem. Biophys. 198, 304-313 (1979).

Kang, M.S., Spencer, J.P. and Elbein, A.D.: The Effect of Showdomycin on Glycolipid Formation. Inhibition of Glucosylphosphoryl-dolichol in Aorta and stimulation of Glucosyl Ceramide in Yeast. J. Biol. Chem. 254, 10037-10043 (1979).

Phillips, L.A., Pang, R.H.L., Park, J.J., Hollis, V.W., Jr., and Famuyiwa, F.: Poly(U)-agarose affinity chromatography: specificity, sensitivity, selectivity, and affinity of binding. Prep. Biochem. 10, 11-26 (1980).

Phillips, L. A. and Kang, M. S.: Structural components of retroviruses: viral RNA and DNA. In Phillips, L.A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker Inc. 1980 (In Press).

Elbein, A.D., Heifetz, A., Kang, M.S., Spencer, J.P. and Keenan, R.W.: Inhibition of Lipid-Linked Saccharide formation by Antibiotics. New York, Academic Press. 1980 (In Press).

Phillips, L. A. and Kang, M. S.: Retroviruses-experimental basis for a molecular approach to human cancer. In Phillips, L.A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker Inc., 1980 (In Press).

Phillips, L. A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker Inc. Publishers, 1980 (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04850-08-LVC

PERIOD COVERED

September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of RNA Tumor Virus Gene Expression in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. S. Nomura	Microbiologist	LVC, NCI
P. J. Fischinger,	Head, Virus Control Section	LVC, NCI
J. W. Hartley	Research Biologist	LVD, NIAID
C. F. T. Mattern	Medical Director	LPD, NIAID

COOPERATING UNITS (if any)

R. H. Pang, Research Associate Scientist, Microbiology Department, New York University Medical Center, New York

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Virus Control Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Northern transfer analysis of viral-specific RNA from one of transformed clones of Moloney murine sarcoma virus infected mouse 3T3 FL S+L- cells and revertant subclones demonstrated the presence of several RNA size classes, 30S, 25 to 23S and 21S. The 30 S RNA was lost from cellular RNAs of the rescue-negative revertants. The largest viral-specific RNA present in Moloney murine leukemia virus producing mouse cells was 35S.

PHS-6040
(Rev. 10-79)

Project No. Z01-CP-04850-LVC

Project Report

Objectives:

To investigate the mechanism of cell transformation and reversion in malignant process.

Methods Employed:

Biochemical and biophysical procedures such as isotopic labeling, isolation of nucleic acids, analytical and preparative ultracentrifugation, reverse transcriptase assays, preparation and charcterization of cDNAs, and nick-translated DNA probes, nucleic acid hybridization, determination of thermal stability of hybrids, chromatography, gel electrophoresis, cleavage of DNA with restriction endonucleases and Southern and Northern transfer technique were employed.

Major Findings:

Employing both complementary DNA probes and DNA probes nick-translated from cloned DNAs, Northern transfer analysis of cytoplasmic RNAs from one of M-MSV transformed clones of mouse 3T3FL S+L- (3B-1-1) cells and revertant subclones demonstrated the presence of several classes of viral-specific RNAs, 30S, 25 to 23S and 21S. The 30S RNA was lost from cellular RNAs of the rescue-negative revertants. In both a phenotypically flat revertant and a rerevertant from which an MSV genome could be rescued, the 30S RNA was transcribed as in the parental S+L- cells. These data further indicated that the loss of the transfomed phenotype did not always reflect changes in transcription of all or part of the MSV genome or the processing of viral-specific RNA.

By means of the Hirt procedure, supercoiled M-MSV (3B-1-1) DNA was extracted from NRK cells producing rat leukemia pseudotype of M-MSV. The DNA demonstrated a unique site for digestion by restriction endonuclease Sac I. Therefore, it may be possible to perform molecular cloning of this strain of M-MSV DNA in λ gtWESλB which contained repeat sequences of Harvey sarcoma genome in EK 2 host.

From August, 1979 to January, 1980, I have learned the following techniques under Dr. J. Alwine of Dr. G. Khoury's laboratory: Northern transfer technique, nuclease S-1 analysis, molecular cloning of SV40 genome, In vitro translation, sequencing of DNA and RNA. At present, in collaboration with Dr. G. Khoury, I am carrying out experiments on genome expression by SV40 using a variety of mutants.

Proposed Course:

Restriction endonuclese analysis of provirus, characterization of virus-specific RNA and investigation of specific gene products in revertants and rerevertants are planned in order to understand the origin of M-MSV and the

nature of both transcriptional and translational restrictions.

Significance to Biomedical Research and the Program of the Institute:

Studies of the cellular and viral functions involved in reversion, the nature and mechanisms of restriction of viral expression by cells, and the genetic analysis of viral DNA may provide an efficient approach to the understanding of viral oncogenesis and host cell control mechanims.

Publications:

Nomura, S.: Envelope properties of murine sarcoma virus-zero (MSV-Z). Virology 101: 300-303, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1-CP-04868-05-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Generation of New Transforming Mouse Type-C Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	U. R. Rapp	Visiting Scientist	LVC	NCI
OTHER:	G. J. Todaro	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)
Dr. Yuan Fon Lee, Bishop College, Dallas, Texas; Dr. Paul O'Donnell, Dr. Nancy Famulari, Memorial Sloan-Kettering Cancer Center, N.Y.; Dr. Sisir Chattopadhyay, Pediatric Oncology Branch, Division of Cancer Treatment, NCI

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were made to isolate and characterize "transforming genes" incorporated into the genome of non-transforming type-C viruses and to employ these new viruses to develop immunological reagents against the products of their transforming genes. New variant murine type-C viruses were isolated and used to define mechanisms for potentiation or attenuation of viral strains. Findings included (a) differences in infectivity between low and high leukemia viruses were directly related to leukemogenicity, (b) the major biochemical difference was within the envelope-gene region and affected the antigenicity of the major viral envelope glycoprotein, (c) leukemogenicity of nondefective viruses seems to be a function of replication efficiency, (d) the susceptibility of cells to infection determined the target tissue for transformation by rapid replicating viruses, (e) the chromosome residence was identified for the receptor-gene code for various classes of viruses, including the new isolates, (f) transplanted tumor cells induced by certain viruses did not grow at the site of inoculation but "homed" into the specific tissue in which it arose originally showing a dependence on that hormonal or tissue factor microenvironment.

Project Description

Objectives:

To isolate and characterize "transforming genes" that have been incorporated into the genome of non-transforming type-C viruses and to employ these new viruses for the development of immunological reagents directed against the products of their transforming genes.

Methods Employed:

Directly transforming retroviruses generally are characterized by the presence of cell-derived, transformation specific sequences that have been incorporated into the retroviral genome. We have developed an in-vitro system that has allowed, for the first time, the systematic isolation of new and tissue specific transforming type-C viruses. We have developed techniques for the derivation of highly infectious forms of these viruses in cell culture. This procedure involves continued selection of the most rapidly replicating progeny virions arising from a series of successive infections of highly permissive host cells. Cell lines from mice which do not produce type-C virus spontaneously or after treatment with halogenated pyrimidines can be activated for virus production by repeated mutagenesis. The most effective mutagen used was ethylnitrosourea (ENU). One genetic change that is commonly observed in MuLV isolates selected for increased oncogenicity in-vivo or in-vitro is a recombinational exchange in the envelope gene. Such viruses infect cells by attachment to a cell surface receptor that is different from the one used by the parental MuLV. Since some of the recombinants with increased oncogenicity, stimulate infected cells to growth in agar, we examined this new receptorbinding activity to determine whether its presence might be the basis of the mitogenic and transforming activity of the recombinant oncogenic viruses.

Major Findings:

A. Virulence of Endogenous Ecotropic MuLV as a Determinant of Leukemia Incidence in Inbred Mice.

The first goal was to test the hypothesis that the virulence of endogenous ecotropic MuLV in inbred mouse strains is a major determinant of leukemia incidence in these strains. We demonstrated that the difference in infectivity observed between BUdR activated ecotropic MuLV from the low leukemia mouse strains C3H/He as well as BALB/c and the high leukemia strain AKR correlates directly with a difference in their leukemogenicity. C3H/He MuLV and BALB/c MuLV from non-transformed cells was poorly infectious and induced 0-5% leukemia late in life whereas ecotropic, XC positive AKR MuLV induced 60-80% leukemia in inbred, leukemia negative NSF/N mice (Rapp, U. R., J.

Virol. submitted for publication, 1980). The major biochemical difference between these viruses that we have identified so far was within the envelope gene region and affects the antigenicity of the major envelope glycoprotein of these viruses. The pathogenic MuLV from AKR has a glycoprotein carrying the antigenic determinants of GIX, a differentiation antigen, whereas the poorly infectious, less or nonpathogenic C3H and BALB n-ecotropic MuLV is negative for this antigen (O'Donnell et al., J. Exp. Med. submitted, 1980).

B. Use of Heterologous MuLV from Asian Mice for the Generation of Oncogenic Variants.

Having established that leukemogenicity of nondefective MuLV appears to be a function of its replication efficiency, we asked whether there was any virus-specific function involved in the transformation process. We adapted a type-C virus from an Asian species of Mus, the M813 virus from M. cervicolor, to high titered growth in NIH 3T3 cells and then tested its leukemogenicity in NSF/N mice. This work (in collaboration with Dr. R. Callahan) succeeded in selecting a rapid leukemia inducing isolate which is presently being examined with respect to possible genomic contributions from NIH mouse cellular genes that might be responsible for its oncogenicity. The parental M813 virus has also induced neoplasia in tissues other than the lymphatic system. The chromosomal DNA of these tumors is being examined by restriction analyses and Southern blotting to determine whether tumors in specific tissue are characterized by the presence of integrated virus in specific chromosomal sites. The fact that M813 virus has very little homology to M. musculus DNA will be a significant advantage in this analysis.

C. Basis for the Tissue Tropism for Transformation by Oncogenic MuLV.

Endogenous C3H MuLV was adapted to high titer growth in fibroblast cells in-vitro. The most common tumors induced by this virus in-vivo, however, were lymphomas rather than sarcomas. As a first step in analyzing the basis of this tissue tropism for transformation by MuLV, we determined the tissue distribution of cell surface receptors required for infection by ecotropic MuLV. Binding experiments with ^{125}I-labeled viral envelope glycoprotein gp70 and cells freshly prepared from all major mouse tissues showed that lymphoreticular tissues had the largest number of free receptors followed by brain and lung. Thus we concluded that susceptibility of cells to infection did determine the target tissue for transformation by rapid replicating MuLV. Yet, preference for transformation of a particular lymphoid cell type may still be a virally determined function. The in-vivo experiments with xenotropic plaque purified clonal isolates of rapid replicating MuLV showed a great variability in the location of the resulting virus induced lymphoid tumor (thymus, spleen, a singular lymph node, liver, kidney or a combination of these tissues). Using clones of hybrids between Chinese hamsters and mouse cells we previously showed that receptors for different classes of endogenous mouse type-C viruses were coded by independently segregating genes, a chromosome 5 coded gene for one class of ecotropic MuLV, a chromosome 2 coded gene for another class of ecotropic MuLV, and still another gene for

receptors used by xenotropic MuLV. In typing the new oncogenic MCF class MuLV that we isolated, we found that several of the leukemogenic isolates used a chromosome 2 coded receptor for infection of fibroblast cells in-vitro. Since some of these envelope recombinants do have the capacity to stimulate infected cells to grow in soft agar it may be possible that some of the cell surface receptors used for infection by MuLV might also function as mitogen receptors. The tissue distribution of such specific classes of receptors might then determine the target cell for transformation by a given recombinant MuLV. We are currently investigating this possibility.

In order to maintain a tumor induced by a newly isolated cell culture derived transforming virus, we generally transplant it in syngeneic weanling mice until we have an established tumor cell line derived from it. These transplantation experiments commonly showed that a particular virus induced tumor did not grow at the site of inoculation but homed into the specific tissue in which it arose originally, where it then grew into a new tumor. Thus it appeared that the tumor inducing virus might have altered the responsiveness of a particular type of infected cell to growth stimulation by factors typical for the microenvironment of spleen or liver or thymus, etc. The molecular basis for an increased responsiveness of infected cells to a given growth factor presumably would have to be sought again amongst the properties of the envelope gene product which might have different functional domains, some acting as cell surface receptor binding sites and others as receptors for tissue specific polypeptide-growth factors. Consistent with this speculation is the finding that certain anti-MCF MuLV antibody preparations have a pronounced growth stimulatory and transforming effect on cells infected with such recombinant MuLV.

D. Cell Culture Derived Directly Transforming Type-C Virus.

The above observations are relevant only for relatively weakly transforming variant MuLV which induce tumors with a latency greater than two months. Very rapidly transforming type-C virus (tumor formation within weeks) such as the established laboratory strains of MSV and some of our more recent cell culture derived isolates transform infected cells instantly to full autonomy of growth. In the animal, such transformed cells have a pronounced tendency for metastasis and, as was first shown in this laboratory, they produce and relase growth stimulating and transforming polypeptides. We have recently isolated in cell culture transforming type-C viruses which induce infected fibroblasts or epithelial cells to growth in soft agar and form sarcomas or carcinomas, respectively in the animal. These isolates behave similar to other laboratory strains of MSV in that some of them are defective for replication, induce nonproducer transformed cells which contain a virus coded polyprotein and rescued virions obtained from such cells do contain an unusual phosphoprotein. We are presently characterizing the genome of one of the new carcinoma inducing isolates after cloning integrated provirus from nonproducer transformed cells in bacteriophage lambda.

Significance to Biomedical Research and the Program of the Institute:

These studies will facilitate detection, isolation, and characterization, of new virogenes and oncogenes, and provide knowledge concerning the regulation of their expression and their involvement in normal development and malignancy. These techniques are directly applicable to type-C viruses of primates and therefore clearly applicable to the research of human cancer.

Proposed Course:

Isolation of new cell derived transforming functions from mouse and human cells by use of non-transforming type-C virus. Analyses of the genes coding for such functions after molecular cloning in a phage vector. Investigation of the gene products of these genes by immunoprecipitation, generation of monoclonal antibodies and functional analyses. The functional analyses will include assay for protein kinase activity and growth promoting activity for various differentiated cells in culture. Experience gained in selecting effectively replicating MuLV virions from noninfectious particles will be applied to the task of stabilizing occasionally produced reverse transcriptase containing particles from human tumor cell lines and selection of infectious human type-C viruses.

Adaptation of the technology developed to the selection of primate type-C virus variants that will indicate the presence or availability of virogenes or oncogenes that interact with other viral information and/or chemical carcinogens or co-carcinogens.

Continuation of testing the potential mechanism of transformation in-vitro in assays in which we will prime soft agar with tissue specific growth factors such as erythropoietin or thymopoietin and test fibroblast cells infected with splenic or thymic leukemia inducing virus for colony growth.

Characterization of the genome of one of the new carcinoma inducing isolates will be accomplished after cloning integrated provirus from nonproducer transformed cells in bacteriophage lambda. The virus-coded phosphoprotein and the question as to whether a virus-specific transformation-specific protein kinase may be present in the transformed cells will be examined.

Publications:

Reynolds, R., Rapp, U. R., Todaro, G. J., and Stephenson, J. R.: Replication-defective S+L-Moloney-MSV codes for an envelope glycoprotein with Moloney- and-MCF-MuLV specific determinants. Virology 99:582-588, 1979.

Sen, A., Halverson, D.O., Rapp, U. R., and Todaro, G. J.: Sarcoma virus-specific phosphoproteins are packaged in "rescued" type C virions. Virology 92:245-251, 1979.

Marshall, T. H., and Rapp, U. R.: Genes controlling receptors for ecotropic and xenotropic type C virus in Mus musculus. J. Virol. 29:501-506, 1979.

Rapp, U. R.: Isolation of leukemia, sarcoma and carcinoma inducing viruses from mouse fibroblast cell lines chronically infected with endogenous MuLV. Cold Spring Harbor Meeting on "Viruses in Naturally Occurring Tumors", Vol. 7, Sept. 1979.

Rapp, U. R., and Todaro, G. J.: Generation of oncogenic mouse type C viruses: In vitro selection of carcinoma-inducing variants. Proc. Natl. Acad. Sci. (USA) 77:1-5, 1980.

Keski-Oja, J., De Larco, J.E., Rapp, U.R. and Todaro, G.J.: Murine sarcoma growth factors affect the growth and morphology of mouse epithelial cells. J. Cell. Physiol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04883-08-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cancer Immunodiagnosis. Cellular Mechanisms of Carcinogenesis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
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INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Human breast cancer specific skin test materials of increased sensitivity were successfully tested in human breast cancer patients. 2. A new bioassay for tumor promoters and carcinogens in mammals was developed, consisting of acceleration of tumor development from Balb/3T3 cells implanted s.c. attached to plastic plates. 3. Methodology was improved for performing a human tumor cloning assay for screening anticancer drugs using the patient's own tumor. 4. Multiple differentiated neoplastic patterns such as chondrosarcoma and malignant pericytoma were identified in tumors produced from Balb/3T3 cells, evidence that the Balb/3T3 cell line is comprised of multipotential embryonic mesenchymal cells. 5. Spontaneous neoplastic transformation in vitro was developed as a model for the in vivo phenomenon of foreign body (smooth surface) sarcomagenesis.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. To develop improved human cancer skin test antigens.

2. To elucidate the mechanisms of carcinogenesis at the cellular level.

3. To identify attributes of tumor cells specifically responsible for malignant behavior.

Methods Employed:

Tumor-graft rejections assay, delayed hypersensitivity assays against tumor antigens, in vitro assays for humoral and cell-mediated immunity against tumors. Cytogenetic procedures. Radioisotopic tracer techniques. Tissue culture and cinematographic procedures associated with tumorigenicity in vivo. Protein biochemistry procedures.

Major Findings:

1. Skin testing human cancer patients with "virus-augmented" crude membrane extracts prepared from cultured human tumors.

We demonstrated in a total of 40 cancer patients that crude membrane (CM) extracts from three different human melanoma lines, infected with vesicular stomatitis virus (VSV) and UV inactivated, gave markedly more sensitive and specific cancer skin test reactions than membranes from non-virus-infected cells. Positive skin tests occurred in 17 of 29 (85%), 11 of 20 (55%), and 13 of 18 (72%) tests, respectively, performed in 20 melanoma patients. The same CM extracts that had not been infected with virus gave positive skin tests in 2 of 20 (10%), 4 of 20 (20), and 2 of 18 (11%) tests, respectively. The three virus-augmented extracts were positive in less than 6% of tests in control subjects with other cancers. The most promising of clinical studies conducted after this initial positive series has been with virus-augmented CM extracts of cultured breast cancer cells. Whereas 14 of 33 (42%) breast cancer patients gave positive skin tests with material prepared from 5 different breast cancer lines, only 3 of 33 (10%) patients with another tissue type of cancer (lung or melanoma) gave positive tests. One breast canceer line in particular, MCF-7, gave positive skin tests in 7 of 7 breast cancer patients, and in only 1 of 7 patients with other cancers.

Use of Paraformaldehyde-Fixed Tumor Cells in Skin Testing. Commercially produced formaldehyde solutions invariably contain approximately 15% of methanol by volume to slow the spontaneous oxidation of formaldehyde to formic acid. A significant number of publications on the fixation of cells with formaldehyde fail to give the commercial source of the reagent and therefore raise the uncertainty that some of the effects could have been due to the presence of methanol. We found in studies of cell-surface binding of

radiolabeled antibodies that commercial formaldehyde-methanol mixtures were much more destructive to tumor cell surface antigens than fresh solutions of pure formaldehyde (a gas, incidentally) prepared by allowing the polymer paraformaldehyde to depolymerize in water. We therefore performed a systematic study to determine optimal conditions for fixation with paraformaldehyde. We found that the cell-mediated immunogenicity of tumor cells in animals was preserved as well as it is with x-ray inactivated cells if fixation with 1% paraformaldehyde was carried out at 37°C. Fixation at room temperature resulted in lowered immunogenicity, and fixation below 10°C practically abolished it. Interestingly, the abrogation of tumor immunogenicity which occurred with fixation below 10°C was reversible. If a portion of the same cells fixed below 10°C were brought to 37°C before fixation, the TATA activity was fully restored. A clinical study is underway to test paraformaldehyde-fixed, virus-augmented and non-virus-augmented, whole tumor cells as a skin test material in cancer patients.

Significance: The very large number of clinical immunotherapy studies with essentially negative results are underscoring the need to return to first principles and ask, "Do the common tissue types of human cancer have tumor antigens at all, and if so, how frequently do they cross-react?" The cell-mediated immune response manifested by skin test antigens offers an efficient way to answer this question.

2. Development of a New Bioassay for Tumor Promoters and Carcinogens: Acceleration of Tumor Development from Balb/3T3 Cells Implanted Subcutaneously Attached to Plastic Plates.

Treatment of Balb/3T3 cells with the promoter phorbol myristate acetate or the mutagen 5-azacytidine before planting on plastic plates increased the incidence of tumors and shortened their latent period. By contrast, the non-promoter 4-alpha-phorbol, 12-13 didecanoae had no tumor accelerating effect. The following table gives some of the data:

Cell line and treatment	Tumor latent period (weeks)		Tumor incidence
3T3	16.4	4.6	50%(15/31)
3T3 plus 10-6 azacytidine	12.2	2.6	90%(9/10)
3T3 plus 10-5 azacytidine	9.6	2.3	80%(8/10)
3T3 plus phorbol myristate acetate	13.7	.45	81%(34/42)
3T3 plus phorbol	13.7	.45	83%(35/42)
3T3 plus 4-alpha-phorbol, 12-13 didecanoate*	16.4	1.1	52%(22/42)

*Non-promoter homologue of phorbol myristate acetate.

Significance: A plate-implant of syngeneic preneoplastic cells is a site of cellular dysplasia that is prone to progress to neoplasia on a predictable

time table. The acceleration of tumor development from plate-implants in animals given carcinogens can be interpreted on the same basis as the earlier appearance of carcinoma of the lung in persons with pulmonary silicosis who smoke heavily. We are in the process of testing authentic human carcinogens of known potency in the plate-implant bioassay using Balb/3T3 cells. Early passage embryo cells are also being developed for use in the bioassay.

3. Optimization of the Human Tumor Cloning Assay for Screening Anti-Cancer Drugs.

The following transplantable human cancers are now being set up in nude mice to provide human tumor material on a predictable and systematic basis: teratocarcinoma, melanoma, lung, and breast. Until this production system becomes available, the Raji suspension culture cell line of human lymphoma cells is being used to examine tissue culture parameters of the cloning assay. We have found that coplating the Raji tumor cells with trypsin-disaggregated mouse embryo cells in soft agar almost doubles the cloning efficiency (the mouse embryo cells do not grow in this system). Raji cells seeded at 103 per plate:

Mouse embryo cells seeded	No. of colonies per plate	% colonies
0	293	100.0
104	332	113.3
105	454	154.9
106	533	181.9

In addition, the presence of the mouse embryo cells result in the colonies of Raji lymphoma cells appearing more compact and spherical, and therefore more easily countable.

Significance: An improved human tumor cloning assay for screening anti-cancer drugs against the patient's own tumor has significance beyond the obvious advantage to the cancer chemotherapist. If this assay could be made a routine part of the medical oncologist's armamentarium, it would provide a broader base for the clinician to do research on the cell and molecular biology of human tumors. In addition, the availability of the nude mouse introduces the potential that at the time of the in vitro assay, the clinician would also be able to characterize the growth behavior and response to therapy of the patient's tumor at the tissue and organ level.

4. Multiple Differentiated Neoplastic Patterns in Tumors Produced by Balb/3T3 Cells Implanted Subcutaneously Attached to Plastic Plates.

Histological section of 96 tumors derived from plate-implants of Balb/3T3 cells showed foci of differentiation into malignant hemangiopericytoma from the capillary pericyte, chondrosarcoma from the chondrocyte, granulocytic

sarcoma from the myeloblast, and fibrosarcoma from the fibroblast. The diagnosis of malignant hemangiopericytoma was confirmed by an internationally known specialist in the histopathology of soft tissue tumors, Dr. Edward H. Soule, at the Mayo Clinic, Rochester, Minnesota.

Significance: It appears that the Balb/3T3 clone A31 cell is a multipotential embryonic mesenchymal cell which is capable of differentiating into a number of connective tissue cell types including the fibroblast. This finding should reorient the interpretation of experiments on Balb/3T3 and C3H/10T 1/2 cells in relation to their biological significance in the adult animal. When any property of these cells, e.g., membrane transport, microfilament distribution, motility, character of the cell cycle, etc., is characterized, the information cannot be directly applied to any cell in adult tissue, including the fibroblast. Our results call attention to the capillary pericyte, identified in the older literature as the Rouget cell. It occurs in the pericapillary region in close association with the capillary endothelial cell and smooth muscle cell (see drawing). All three of these cell types possess a basal lamina and intermediate and tight junctions. The pericyte appears to be a stem cell that can differentiate into a fibroblast endothelial cell, smooth muscle cell, and possibly other cell types. Franks has concluded that many mouse and human cell lines initiated from normal tissue are ultimately overgrown by the pericytes and endothelial cells of small blood vessels. Brand believes that sarcomas of mice produced by the subcutaneous implantation of plastic film may be derived from the capillary perictye.

5. Spontaneous Neoplastic Transformation In Vitro of Connective Tissue Cells as a Model for the In Vivo Phenomenon of Foreign Body Smooth Surface Sarcomagenesis.

Spontaneous neoplastic transformation in vitro: Rodent fibroblasts and embryo cells maintained in tissue culture routinely undergo spontaneous neoplastic transformation after 100 to 300 days. The etiology of this phenomenon has never been determined. A large body of literature has shown that it occurs in the absence of serum in the culture medium, in the absence of light, and in a wide variety of tissue culture media. It occurs in mouse connective tissue cells placed in millipore membrane diffusion chambers and implanted intraperitoneally. In this last case of "tissue culture in vivo", every parameter of tissue culture except attachment to a smooth surface was excluded.

We postulated that just as juxtaposition in vivo between the smooth surface of a plastic film implant and subcutaneous connective tissue gives rise to sarcomas, so juxtaposition in vitro between the smooth surface of the tissue culture vessel and explanted connective tissue cells could also give rise to sarcomas by a similar mechanism. This postulate is more readily acceptable when one views the behavior of connective tissue cells in vitro in relation to their pathophysiological role in vivo. When connective tissue cells (mostly fibroblasts and endothelial cells) are explanted into tissue culture, they react as they would if confronted by an acute traumatic wound in the host produced by a smooth surfaced foreign body, e.g., a plastic knife. In such a

wound in vivo, contact with the foreign smooth surface and exposure to the mitogenic serum factors generated by hemorrhage and clot formation activate the normally cigar-shaped, non-motile, non-proliferative fibroblast to convert to an enlarged, flat, adherent, highly motile and rapidly proliferative cell that sets about "walling off" the foreign body by forming a multilayered capsule around it. In the in vitro situation, the fibroblast reacts to the bottom of the petri dish as though it were a smooth-surfaced foreign body, and to the mitogenic factors in the serum of the culture medium as though acute hemorrhage with clot formation had occurred nearby. With this perspective in mind, that the fibroblast in vitro behaves according to its encoded epigenetic programs of wound repair and foreign body rejection, the conventional in vitro properties of density inhibition of proliferation, anchorage dependence, and serum growth requirement can be compared with their in vivo counterparts as follows:

IN VITRO (TISSUE CULTURE)	IN VITRO (WOULD BY SMOOTH FOREIGN BODY)
DENSITY DEPENDENT INHIBITION PROLIFERATION. Growth slows rapidly proliferating fibroblasts form a multi-layer on the surface of the culture vessel.	DENSITY DEPENDENT INHIBITION OF PROLIFERATION. Growth slows as rapidly proliferating fibroblasts form a multilayered capsule around a smooth-surfaced foreign body.
ANCHORAGE DEPENDENCE. Cells "require" attachment to a solid surface to flatten, move, and proliferate.	FOREIGN SURFACE ACTIVATION. Cells are activated by contact with a smooth-surfaced foreign body to convert from a cigar-shaped, dormant cell to a flat, motile, proliferative cell.
SERUM GROWTH "REQUIREMENT". Cells require the presence of serum to grow "normally".	SERUM ACTIVATION. Serum produced by hemorrhage and clot formation contains factors which stimulate fibroblast growth during wound repair.

Evidence that foreign body smooth surface sarcomagenesis is closely related to spontaneous neoplastic transformation in vitro. We used a two-step experimental procedure. For the first step, we explanted and grew mouse subcutaneous cells in vitro, thereby exposing them to the smooth surface of the tissue culture flasks. For the second step, at various times we tested the cultured cells for tumorigenicity by implanting them subcutaneously at relatively low cell dosage (104 - 105 cells) when they were attached to plastic plates measuring 0.2x5x10 mm. After only 21 to 30 days in vitro, cells produced tumors in 18 of 192 implants (9.3%). After 3 to 4 months in vitro, cells produced tumors in 14 of 50 implants (28%). Plates alone never produced tumors after 15 months of observation (the size of the plates is just

below the minimum required for foreign body smooth surface sarcomagenesis). In these experiments, the cultured cells were never tumorigenic when inoculated in fluid suspension in doses as high as 1x107 cells, even after 134 days in culture. There was an inverse correlation between the time spent by the cells in culture and the latent period before appearance of sarcomas after the cells had been implanted attached to plastic plates. The latent period varied from 26 to 79 weeks. In addition, the frequency of tumors correlated directly with time in tissue culture. It appears that the attachment of connective tissue cells to the surface of the culture vessel per se at least promotes the preneoplastic progression of the cells, and may even initiate the neoplastic process through factors related to the geometry of stretching and attachment or the physiology of motility and mitosis in the stretched adherent state. In consideration of the above experimental findings and a voluminous literature which excluded other tissue culture parameters as primary causal agents of spontaneous neoplastic transformation in vitro, the hypothesis is strengthened that contact of the cells with the surface of the culture vessel per se constitutes a major causal mechanism and that this mechanism is related to the one which operates during foreign body smooth surface sarcomagenesis in vivo.

Significance: The above accumulation of evidence and ideas tends to substantiate use of the phenomenon of spontaneous neoplastic transformation in vitro of connective tissue cells as an in vitro model for foreign body smooth surface tumorigenesis. The model is remarkably manipulable and quantifiable, and the very earliest changes in the neoplastic process are accessible to delineation. In preliminary studies we have found that primary explants of mouse connective tissue at high dilution produces clones that can be classified into distinctive morphologic types. We intend to identify the in vivo cells that produce these clonal types, and to analyze the change in frequency, morphology, and behavior of the clonal types as the culture population of connective tissue cells is carried through the stages of preneoplastic progression and neoplastic conversion. The process of foreign body smooth surface sarcomagenesis will also be studied by clonal analysis of cells present in the capsule surrounding a subcutaneously implanted plastic plate. Comparisons between the in vivo and in vitro systems of neoplastic progression will be easily possible and illuminating.

Significance to Biomedical Research and the Program of the Institute:

See discussions of Significance above.

Proposed Course:

Continuation of project areas described.

Publications:

Austin, F.C. and Boone, C.W.: Virus agumentation of the antigenicity of tumor cell extracts. Adv. Cancer Res. 30:301-344, 1979.

Oikawa, T., Gotohda, E., Austin, F.C., Takeichi, N. and Boone, C.W.: Temperature-dependent alteration in immunogenicity of tumor-associated transplantation antigen monitored via paraformaldehyde fixation. Cancer Res. 39:3519-3523, 1979.

Boone, C.W., Vembu, D., White, B.J., Takeichi, N. and Paranjpe, M.: Karyotypic, antigenic, and kidney-invasive properties of cell lines from fibrosarcomas arising in C3H/10T 1/2 cells implanted subcutaneously attached to plastic plates. Cancer Res. 39:2172-2178, 1979.

Boone, C.W. Takeichi, N., Eaton, S. and Paranjpe, M.: "Spontaneous" neoplastic transformation. Science 204:177-179, 1979.

Sanford, K.K., Boone, C.W., Merwin, R.M., Jones, G.M. and Garrison, C.V.: The plate-implant as a bioassay for the neoplastic potential of cultured cells. Int. J. Cancer, April, 1980.

Boone, C.W. and Scott, R.E.: Multiple differentiated neoplastic patterns in tumors produced by Balb/3T3 cells implanted subcutaneously attached to plastic plates. J. Suprmolec. Struc. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04916-04-LVC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Organization and Evolution of Endogenous Mammalian Type C Viral Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	G. J. Todaro	Medical Officer	LVC	NCI
	E. Birkermeier	Staff Fellow	LVC	NCI
	N. Battula	Visiting Associate	LVC	NCI
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None

LAB/BRANCH Laboratory of Viral Carcinogenesis

SECTION Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The distribution within primates of nucleic acid sequences related to endogenous retroviruses, their sequence organization and the factors influencing their biological activity was studied. Specifically, (1) to detect and characterize the virus-related sequences in primates and the factors involved in their evolution; (2) to develop probes appropriate for the detection of virus-related sequences in human cells; (3) to characterize the organization of the 50-100 copies of endogenous sequences through restriction mapping and recombinant DNA techniques; (4) to correlate the extent of viral expression with the state of integration of viral sequences, both endogenous and exogenous, through restriction mapping and transfection; (5) to investigate other factors involved in the expression of viral sequences in primates.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To characterize the distribution within primates of nucleic acid sequences related to endogenous retroviruses, their sequence organization and the factors influencing their biological activity. Specifically, (1) to characterize the virus-related sequences in primates and the factors involved in their evolution; (2) to develop probes appropriate for the detection of virus-related sequences in human cells; (3) to characterize the organization of the 50-100 copies of endogenous sequences through restriction mapping and recombinant DNA techniques; (4) to correlate the extent of viral expression with the state of integration of viral sequences, both endogenous and exogenous, through restriction mapping and transfection; (5) to investigate other factors involved in the expression of viral sequences in primates.

Methods Employed:

The existence of endogenous viruses indicates that similar viral genomes or related sequences may exist in human cells. Since we would ultimately like to characterize such human viral sequences we have focused on the viruses isolated from man's closest relatives, the Old World monkeys. These viruses fall into three distinct classes: (1) the various closely related isolates of type-C virus from several species of baboon (we deal with the M7 and M28 isolates), (2) a second type-C class which includes MAC-1 from stumptail macaque, CPC-1 from colobus and a related virus from rhesus and (3) the type-D virus, LAD-1, isolated from langur. Irrespective of their direct relationship to a putative human virus, these viruses compose a useful system in which to study the general characteristics of endogenous viruses. The primate system parallels the avian and rodent systems but has somewhat different characteristics from the other two systems which may be related to the fact that all the present primate viruses isolates are xenotropic.

Major Findings:

A. Detection of Virus-related Sequences in Primates and Their Evolutionary Characteristics.

We have used very low stringency hydroxyapatite techniques in order to detect sequences which are distantly related to the three classes of Old World primate viruses and to quantitate their sequence divergence. Sequences related to both type-C families diverge more rapidly in Asian primates than in African primates, but the differences between Asian and African primates are greater with the baboon virus family. In both cases, we can detect related sequences in the African apes, chimpanzee and gorilla, but not in the Asian

apes, orangutan or gibbon, nor in man. In contrast, the type-D sequences show no difference between African and Asian primates but diverge so rapidly that we cannot detect related sequences in any of the apes. The evolutionary pattern with all three viral families suggests that related sequences are present in orangutan, gibbon and man, but have diverged enough to escape detection by the present method.

The existence of two isolates of the same family, MAC-1 and CPC-1, but from relatively distant species allows us to construct an evolutionary tree for this family of viral sequences. Comparison of this tree with that derived from fossil evidence and single copy cellular DNA allows us to conclude that horizontal transmission of virus from one branch of the tree to another is not the reason for the difference between the viral sequences of African and Asian primates. Instead, the results support the hypothesis that the rate of divergence accelerates once a species leaves Africa.

The shape of the melting curves of viral cDNA hybridized to cellular DNA of the species from which the virus was isolated demonstrates that the 50-100 copies of endogenous sequence are quite homogeneous. This would not be the case if they had evolved independently for tens of millions of years. By analogy with other homogeneous, multigene families, such as the ribosomal RNA and the histone genes, we expect that the homogeneity of the multiple copies is maintained by a mechanism such as unequal crossing-over. If this were the case, we would expect that the viral copies are clustered with a repeated spacer between the copies. Restriction enzyme analyses of cellular DNA support this hypothesis.

The divergence of single copy cellular DNA between the primate species provides the background information necessary for the evaluation of the evolution of virus-related sequences. Using the low stringency hydroxyapatite techniques, we have extended existing data to include the prosimians. The resulting phylogenetic tree resolves a number of controversies as to the placement of early branch points. We unexpectedly find that the primates on Madagascar have evolved substantially slower than all other primates. This result is not predicted by the theory of neutral selection, but can be explained by several hypotheses based on the 50 million year isolation of Madagascar. Since this result may be of major importance to our understanding of DNA evolution in general, we are planning experiments to determine if carnivores also evolve slower on Madagascar than elsewhere. A positive result would eliminate the rather trivial explanation that the slow down results from a founder effect in which the original Madagascar primates had an abnormally low spontaneous mutation rate.

B. Detection of Endogenous Viral Sequences in Man.

The results to date using representative cDNAs from virus isolates to detect human endogenous sequences have been negative. There are two useful approaches for obtaining more appropriate viral probes. The first one is to

determine whether there are regions of the viral genome that are more conserved than average and whether these regions taken from available viruses will suffice. Data with the strong stop sequence from CPC-1 suggests that strong stop sequences are not more conserved than average. Further dissection of the genome will proceed when we have cloned unintegrated viral DNAs. The second approach is to obtain viral sequences from a closer relative to man. To this end we are in the process of cloning the type-C virus-related sequences from chimpanzee, the closest relative of man for which we can detect related sequences using presently available viral cDNAs.

C. Organization of Unintegrated and Integrated Viral DNA Sequences and Their Relationship to Biological Activity.

Analysis of the unintegrated DNA of baboon endogenous virus (M7) showed two molecular forms. The minor form consisted of two covalently closed circular DNA species of approximately 9.0 Kb and differing from each by about 0.5-0.6 Kb. The smaller of the two was in 2-3 fold excess over the larger species. The circular nature of these two DNA species was substantiated by restriction enzyme analyses. The major form of unintegrated M7 DNA consisted of a single species of linear DNA corresponding to 9.0 Kb and had a 0.6 Kb direct terminal repeat on each end. The linear DNA was biologically active when transfected onto dog cell, bat cell or human cell recipients and the virus produced exhibited properties similar to the parent M7.

A physical map of the infectious linear DNA was constructed by digesting the DNA with different restriction enzymes and locating their sites of recognition on the viral DNA. The enzymes include one no cut enzyme, one enzyme that cuts only in the terminal repeats, and three enzymes that make one cut. The order of these fragments was determined by double digestion and the viral DNA was oriented with respect to the 3' and 5' end of viral RNA. The integrity of the terminal repeats in the unintegrated linear viral DNA as well as in the integrated DNA appears to be essential for biological activity. Removal of part of the terminal repeats by digestion with Xho I which cuts only in the terminal repeats abolished biological activity. However, the untreated DNA as well as the DNA digested with the no cut enzyme retained biological activity. We are using recombinant DNA techniques to clone unintegrated DNA which should allow the determination of the minimum nucleotide sequence necessary for biological activity.

The specific site of the infectious viral DNA used in integration was investigated by restriction analyses of infectious high molecular weight DNA. No detectable permutations of viral DNA sequences compared to the linear map and no significant deletions or substitutions in the viral DNA were found. The sequences in the end region of the terminal repeats of viral DNA were used in fusing with cellular DNA sequences. Analyses of cellular DNA with respect to its sites of integration by restriction enzyme showed that the fusion fragments (consisting of cellular and viral sequences) were unique for each clone and the size of the fragments was different in different

clones. These results show that several sites in the cell DNA can accommodate viral DNAs.

Restriction analysis of the multiple copies of the M7-related sequences in baboon DNA shows that their organization is surprisingly simple. The cellular DNA sequences flanking the endogenous viral DNA sequences are reiterated indicating common sites of residence in cell DNA. The viral sequences appear to be non-permuted and non-tandem. There are also no significant insertions or deletions in the viral DNA except possibly in the terminal repeats. In spite of the similarity of the endogenous sequences to the M7 sequences in infected cells, the endogenous sequences were not biologically active. Further analyses of the organization of the endogenous sequences will be pursued using specific probes prepared from cloned viral DNA. In addition, we plan to use the green monkey genomic library provided by Dr. Maxine Singer to isolate the three classes of viral sequences from a single species and to characterize the flanking sequences.

D. Transcription and Processing of Virus-specific RNAs in CPC-1 Infected Cells.

The expression of primate retroviruses in productively and nonproductively infected cells is being investigated employing RNA blotting procedures. The combination of a poly (A) selection procedure applied to the RNA molecules with the use of a probe specific to the 5' end of the virus ensures that any alterations in the size of these molecules must be the result of post-transcriptional modifications (splicing). The application of this analytic procedure to CPC-1 infected human lung carcinoma cells (A549 cells) revealed two virus-specific RNAs (8.00 Kb and 2.95 Kb) which likely represent the genome-length "gag" and "pol" messages and the spliced "env" message, respectively. Interestingly, following the treatment of these cells with DMSO (a chemical known to modify cellular transcriptional controls) an additional RNA species of 4.5-4.6 Kb in length has been detected which hybridizes to the virus-specific probe. We are presently investigating the origin of this novel RNA, i.e., does it represent an intermediate in the splicing of viral RNAs or the derepression of cellular mRNA which contains sequences related to those found to be common among the retroviruses.

Significance to Biomedical Research and the Program of the Institute:

Our evolutionary data strengths the hypothesis that the three classes of endogenous Old World primate viruses have been genetically transmitted since the early stages of primate evolution. The corollary that such sequences should exist in man is thereby also strengthened. Although actual detection of these sequences still eludes us, the data guides us in the next stages of the search. The first approach will try to dissect the viral genome to define especially conserved regions in the available viruses which might allow detection of related human sequences. The dissection of the viral genome may also provide more general information on viral evolution or on the evolution of cellular repeated sequences. The second approach is to

isolate the virus-related sequences from close relatives of man by molecular cloning techniques.

The quantitative differences in evolutionary patterns for the 3 classes of viruses demonstrates that they have been subject to differing evolutionary pressures and therefore do not play a totally neutral role in the cells. The molecular cloning of viral sequences and their restriction mapping provides us with powerful tools to study their biological activity at the molecular and cellular levels. The results to date suggests that the organization of the endogenous primate viral sequences is simpler than for the murine and avian cases. This probably occurs because the possibility of infection of primate cells with closely related viruses is quite limited.

Proposed Course:

The existence of endogenous viruses indicates that similar viral genomes or related sequences may exist in human cells. Since we would ultimately like to characterize such human viral sequences we have focused on the viruses isolated from man's closest relatives, the Old World monkeys. Irrespective of their direct relationship to a putative human virus, these viruses compose a useful system in which to study the general characteristics of endogenous viruses. The primate system parallels the avian and rodent systems but has somewhat different characteristics from the other two systems which may be related to the fact that all the present primate viruses isolates are xenotropic. For these reasons this project will be continued and extended along the lines already described.

Publications:

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Bonner, T. I., and Todaro, G. J.: The evolution of baboon endogenous type C virus: Related sequences in the DNA of distant species. Virology (in press).

Bonner, T. I., Heineman, R., and Todaro, G. J.: The evolution of DNA sequences is retarded in lemurs and other Malagasy primates. Nature (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04994-03-LVC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Molecular Studies of Mammary Carcinoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☑ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies have involved the delineation of the distribution and diversity of mouse mammary tumor viral (MMTV) and MMTV-related genetic information in rodents and its correlation with the etiology of mammary carcinoma. Major accomplishments during the past year include: (a) the isolation, from the RNA genomes of highly oncogenic MMTVs, of a subset of sequences that are detected (both by liquid hybridization and restriction endonuclease techniques), only in the DNA of mammary tumors that arise early in life of several mouse strains, and the characterization of these tumor associated sequences (TAS); (b) the use of liquid hybridization and restriction endonuclease techniques as an adjunct in the detection of metastatic mammary tumor cells; (c) the delineation of the diversity of mammary tumor viral genes that exists within the genus Mus and the species M. musculus; (d) a demonstration of the diversity of endogenous MMTV genes among colonies of the so-called "inbred" C3H mouse strain and the presentation of evidence for the recent (within the last few decades) de-novo integration of MMTV sequences as endogenous provirus; (e) the first demonstration that the rat genome contains MMTV-related information.

Project Description

OBJECTIVES: To quantitatively and qualitatively define the spectrum of MMTV-specific and MMTV-related proviral information in rodent species and to search for unique genetic elements that are associated with the occurrence of mammary carcinoma. Specific aims include:

1. The isolation and characterization of a subset of sequences obtained from the RNA genomes of highly oncogenic MMTVs, that are detected in the DNA of mammary tumors that arise early in life of several mouse strains.
2. The use of liquid hybridization and restriction endonuclease techniques as an adjunct in the detection of metastatic mammary tumor cells.
3. Delineation of the diversity of mammary tumor viral genes within the genus Mus, the species M. musculus and the "inbred" strain C3H.
4. The characterization and isolation of MMTV-related information in the rat.

BACKGROUND AND RATIONALE: The distribution of MMTV proviral copies has been shown to vary from one mouse strain to another, or from one tumor to another in the same mouse strain. For example, the DNA of C3HfC57BL mice contains 3-4 MMTV proviral copies per haploid genome, whereas the GR genome contains approximately 16 copies. Restriction endonuclease studies have shown that mice display a heterogeneity with respect to both copy number and sites of insertion of MMTV-specific DNA. The rationale of our studies is to track the distribution and diversity of both endogenous and exogenous cellular genes that demonstrate sequence complementarity to known mammary carcinoma inducing viruses. These studies should provide insight as to which events, at the host genome level, either initiate or promote mammary tumorigenesis.

MAJOR FINDINGS:

Isolation and Characterization of Tumor Associated Sequences (TAS) of MMTV(C3H).

C3H/He mice contain at least two MMTVs, one of which [MMTV(C3H)] is highly oncogenic and readily isolated from milk or from mammary tumors arising before one year of age. To determine if there are MMTV proviral sequences present in early C3H/He mammary tumors that are not present in the DNA of late occurring mammary tumors and in apparently normal C3H tissues, recycling experiments were conducted. Iodinated MMTV(C3H) 60-70S RNA was first hybridized to a vast excess of apparently normal C3H liver DNA. The unhybridized single stranded [^{125}I]-RNA was eluted from hydroxylapatite and concentrated. This recycled MMTV(C3H) RNA failed to hybridize to DNA of normal C3H/He tissues but did hybridize extensively to DNA from early occurring C3H/He mammary tumors; this probe has thus been termed tumor associated sequences (TAS). These sequences were shown to be somewhat spread throughout the genome, but are enriched at the 3' end of the molecule. Studies were conducted to determine the natural

distribution of proviral sequences homologous to TAS. Complementary sequences were found in the DNA of all "early" mammary tumors tested of C3H/He, RIII, and DBA mice but not in the DNA of normal tissues of those same mice. Complementary sequences were found, however, in the DNAs of both GR mammary tumors and all GR organs tested. DNAs of "late" occurring mammary tumors of the low and moderate incidence strains BALB/c and C3HfC57BL were also analyzed for the presence of TAS and were consistently found negative. Livers of BALB/c, C57BL/6N, C57BL/10SCN, A/He, NIH Swiss, NZB/N, and NZW/N were shown to contain some MMTV proviral information; they do not, however, contain, sequences homologous to TAS.

We have also employed restriction endonuclease digestion and the Southern DNA transfer techniques to determine the integration pattern and distribution of MMTV provirus in murine cellular DNAs. When DNAs are digested with restriction endonuclease Pst-1, unique internal restriction fragments of 2.5 and 0.6 megadaltons are observed. These fragments are absent in tissues which do not contain TAS in their DNA. It thus appears that the viruses responsible for causing early mammary tumors in C3H, RIII, and GR mice are easily distinguishable from the viruses causing late mammary tumors in C3HfC57BL mice and BALB/c mice by the presence of both TAS, and specific restriction fragments in members of the former group.

The Use of Molecular Hybridization as an Adjunct to the Detection of Pulmonary Metastases in Mammary Tumor Bearing Mice.

The murine model represents an excellent system for the study of metastatic dissemination of mammary tumor cells. Several methods have been used to score for pulmonary metastases; these include gross and microscopic examination of lung tissues, and bioassays by trochar implants of lung tissues into syngeneic mice. Since the radioactively labeled TAS-RNA isolated from MMTV(C3H) hybridized selectively to the DNA of mammary tumor cells, studies were undertaken to determine if this probe could be used as an adjunct in the detection of mammary tumor cells at distal sites, i.e., metastases.

Lung and spontaneous mammary tumors from C3H/He mice were examined by immunoperoxidase staining for MMTV antigens and conventional histologic techniques. All mammary tumors from seven animals examined had immunoperoxidase detectable MMTV antigens. Pulmonary metastases were found in each of the lungs of three animals whose entire lungs were examined. Metastases were detected, however, in only one of four lungs when sampled by random biopsy; three of these four identical lung samples, on the other hand, were positive when assayed by the nucleic acid hybridization procedures described below. Pulmonary metastases appeared as masses within the pulmonary arteries or as expansile masses within the pulmonary parenchyma. The metastatic cells frequently extended through the wall of the blood vessel. There was no evidence of individual tumor cells lodged in blood vessels. No cells outside the tumor mass were positive for MMTV antigens.

When ^{125}I-labeled TAS RNA was annealed to the DNAs of various internal organs of the C3H/He mice bearing spontaneous mammary tumors, six of eight lungs gave a positive hybridization, whereas all hearts, kidneys, brains, and livers scored negative. The lack of detection of TAS in the DNAs of other highly vascularized organs, such as liver, provides evidence that the hybridization to lung DNA is not simply detecting circulating tumor cells. To determine the approximate sensitivity of the hybridization method to detect mammary tumor cells in pulmonary tissue, mixing experiments were performed. Lung cells from apparently normal C3H/He mice were mixed in various ratios with C3H mammary tumor cells, DNAs were extracted, and then hybridized to TAS ^{125}I-RNA. Hybridization above background levels could be detected in ratios of at least one mammary tumor cell per 250 normal lung cells. Recent studies using restriction endonuclease techniques have detected 1 tumor cell per 500 normal lung cells. The nucleic acid hybridization studies reported here conceptually add a new dimension in the detection of metastatic spread of tumors. To our knowledge, it is the first attempt to use the technique of molecular hybridization to such an end. Some of the advantages of this technique include: (a) the high degree of sensitivity, (b) the specificity of the hybridization reaction, and (c) the large amounts of a given tissue that can be tested in one assay. This latter property reduces the possibility of missing metastatic cells in some tissues due to sampling error when only a part of a given tissue is examined histologically.

De-Novo Integration of MMTV Proviral Sequences.

Since the C3H mouse is the most extensively used in virus studies of murine mammary cancer, the distribution of MMTV proviral sequences was examined in several colonies or substrains of C3H mice. C3H mice arose from the Bagg albino X DBA cross and were subsequently distributed to different investigators. When radioactive MMTV(C3H) 60-70S RNA or cDNA was hybridized to individual organs of different colonies of C3H mice, all tissue hybridized to essentially the same extent and with a C_0t 1/2 of approximately 600.

We have shown above that DNAs from apparently normal tissues of C3H/HeN mice are devoid of proviral sequences complementary to MMTV(C3H) TAS RNA. This probe failed to hybridize to DNA from individual organs of 32 apparently normal C3H/St, C3H/StWi, and C3H/HeN mice. The TAS-RNA probe, however, did hybridize to DNAs of all organs tested from 24 animals from the two colonies of C3H/Bi mice (C3H/BiSim and C3H/BifMai). C_0t analyses revealed the MMTV TAS proviral DNA in both C3H/Bi substrains is present at approximately one copy per haploid genome. This copy number is consistent with a recent acquisition of these sequences. We have recently confirmed and extended these studies employing restriction endonuclease techniques. Pst-1 cuts clearly reveal the existence of a 2.5 and 0.6 megadalton bands in C3H/Bi mice that are absent in C3H/He mice.

The C3H mouse was derived by Strong, from the cross of a DBA mouse and a Bagg albino mouse (the progenitor of the BALB/c mouse). In the early 1930s, breeding pairs of C3H/St mice were sent to both Bittner (to form the C3H/Bi substrain) and Andervont, who later passed them on to Heston (to form the C3H/HeN substrain). The C3H/Bi mice studies here were derived from the Bittner colony. The most likely explanation of these results involves the quite recent integration of these sequences. Since feral mice, subspecies of M. musculus, and other Mus species do not contain DNA sequences related to the TAS of MMTV(C3H) RNA, it is reasonable to assume that the presence of these sequences in any given mouse or substrain (i.e., C3H/Bi) is the result of a recent acquisition event, rather than the result of a deletion. This integration may well have occurred within the 15-year interval when these mice were sent to Bittner (circa 1930) and before the progenitors of the C3H/BiSim and C3H/BifMai colonies were separated (circa 1945). The fact that the TAS proviral DNA is present at only one copy per haploid genome in C3H/Bi DNA (compared to four copies in GR mice) whereas the rest of the MMTV genome is integrated at approximately five copies per haploid genome in the same C3H/Bi DNA is also consistent with a recent integration event. Regardless of the biologial explanation for these results, they demonstrate the diversity that may exist in "inbred" strains of laboratory mice commonly used in cancer research. Results obtained by investigators in different laboratories or by the same investigator over a period of several years may, therefore, be subject to the differential segregation of proviral sequences or perhaps to the de-novo integration or reintegration of viral genes.

Differential Distribution of Mouse Mammary Tumor Virus-related Sequences in the DNAs of Rats.

The occurrence of spontaneous mammary tumors in various rat strains ranges from less than 1 to 30% of all tumors. Mammary tumors in the F344 rat have been reported to be as high as 11.5% in males and 19.6% in females. To determine if sequences related to, but not identical to, the MMTV could be detected in the DNA of rats, we relaxed the conditions of hybridization by lowering the temperature at which hybridizations were performed from 68 to 54°C. To assay RNA-DNA duplexes, we raised the salt concentration used for RNase treatment from the standard conditions of 2X SSC to 20X SSC in several increments with maximum hybridization observed at 8X SSC. The percent hybridization obtained between MMTV 60-70S ^{125}I-labeled RNA and DNA from various rat tissues increased from background levels to approximately 17%. Specificity was still maintained; i.e., no hybridization was observed to DNA obtained from guinea pig, hamster, bovine, and canine tissues, as well as to E. coli. Five laboratory strains of rats tested contained MMTV-related proviral information. To determine if this information was endogenous, we examined DNA from each organ of several feral rats; all tissues of all feral rats assayed were identical with C_0t 1/2 values of approximately 800. The MMTV 60-70S [^{3}H]RNA used in these experiments was obtained from

virions from the supernatants of MMTV-infected feline cells. We used this RNA to rule out the possibility that normal murine or feline cellular RNA or DNA was contaminating the MMTV 60-70S RNA preparations. No hybridization was obtained when this RNA was hybridized to the uninfected feline cells. These experiments further demonstrated that the hybridizations observed to rat DNAs were the result of the presence of nucleic acid sequences coded by the MMTV genome.

Whereas the DNA of all laboratory strains and feral rats exhibited approximately 17% hybridization to MMTV 60-70S RNA, DNAs from organs of some F344 rats gave levels of hybridization as high as 30-35%. Of 115 tissues tested from F344 rats, 48 showed this higher level of hybridization. Those DNAs of F344 rats that showed higher levels of hybridization with MMTV 60-70S RNA also selectively demonstrated annealing with MMTV(C3H) TAS probe under relaxed hybridization conditions. The hybrid duplexes formed between MMTV(C3H) 60-70S ^{125}I-labeled RNA and rat DNAs displayed a Tm of 11.6^{o}C., i.e., 5.3^{o}C lower than the homologous Tm. Duplex structures formed between TAS RNA and FRE cellular DNA displayed a Tm of 15.5^{o}C, i.e., a value approximately 8.5^{o}C lower than that for the homologous hybrid structure. These findings thus demonstrate that laboratory strains of rats commonly used in mammary cancer research contain at least one set and perhaps two different sets of MMTV-related information in their DNAs. The first set is present in the DNAs of all rats in the low repetitive range and is related to approximately 20% of the MMTV genome. The second set of sequences, found thus far only in F344 rats, is slightly more diverged from the MMTV genome than the endogenous sequenes.

Studies are in progress to employ relaxed hybridization conditions in restriction endonuclease analyses of rat DNAs. We plan to eventually identify unique rat restriction fragments related to MMTV genomic sequences. Once identified, these fragments will be cloned and used to prepare radioactive probes to determine if the transcription of these sequences is of consequence to the occurrence of naturally occurring mammary neoplasia in the rat.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROPOSED COURSE: These studies have demonstrated the broad spectrum of genetic information that may exist, either as exogenous or endogenous genetic elements, that is complementary to the genomes of known mammary carcinoma inducing viruses. The data presented demonstrate the diversity of these host genomic elements that may even exist within a so-called "inbred" mouse strain commonly used in cancer research. The detection of both endogenous and exogenous genomic sequences, related to those of known mammary tumor inducing viruses, will now permit studies on the possible involvement of viruses in mammary cancer of the rat. These studies being undertaken should provide additional insight as to which events at the genomic level are involved in either the initiation or promotion of mammary tumorigenesis.

PUBLICATIONS:

Drohan, W. and Schlom, J.: Differential distribution of mouse mammary tumor virus-related sequences in the DNA of rats. J. Natl. Cancer Inst. 62:1279-1286, 1979.

Drohan, W. and Schlom, J.: Diversity of mammary tumor viral genes within the genus Mus, the species Mus musculus and the strain C3H. J. Virol. 31:53-62, 1979.

Schlom, J.: Type-B and type-D retroviruses. In Stephenson, J. (Ed.): Molecular Biology and RNA Tumor Viruses, Chapter 11. New York, Academic Press, Inc., pp. 447-484, 1980.

Schlom, J., Colcher, D., Drohan, W., Horan Hand, P., Howard, D., and Teramoto, Y. A.: Systematics of murine mammery tumor viruses. In Brennan, M., Rich, M. A., and McGrath, C. M. (Eds.): Advances in Breast Cancer Research. New York, Academic Press, Inc. (In Press).

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Drohan, W., Young, J., and Schlom, J.: Correlation between the development of murine mammary cancer and the segregation of endogenous genes. In Fields, B., Jaenisch, R., and Fox, C. F. (Eds.): Animal Virus Genetics, ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Academic Press, Inc., Vol. XVIII (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04995-03-LVC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Characterization of Primate Type-D Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of these studies is to characterize (at the molecular and immunochemical level) the type-D retroviruses recently isolated from nonhuman primate and human cells, to define their distribution in primate tissue, and their relationship, if any, to mammary carcinoma. Major accomplishments during the past year include: (a) delineation of the distribution and diversity of type-D retroviral genes in primates and characterization of their mode of transmission; (b) development of novel group and interspecies radioimmunoassays for the detection and classification of type-D retroviruses of several primate species; (c) the detection of type-D retroviral gene products in primate tissues; (d) immunologic characterization of unique type-D retroviruses isolated from human cells.

PHS-6040
(Rev. 10-76)

Project Description

OBJECTIVES: To immunologically characterize the type-D retroviruses recently isolated from nonhuman primate and human cells, to define their distribution in primate tissues, and to determine their relationship, if any, to mammary carcinoma. Specific aims include:

(a) Determination of the distribution and diversity of type-D retroviral genes in primates.

(b) Development of group and interspecies radioimmunoassays for the detection of type-D retroviral gene products and employing these assays to determine if related translational products are found in human tissues.

(c) Characterization of type-D retroviruses reported to be isolated from human cells.

BACKGROUND AND RATIONALE: Type-D retroviruses have now been isolated from at least three primate species. The various isolates are distinct morphologically from type-B and type-C retroviruses and have, therefore, been classified separately. They do, however, share some features in common with the type-B retroviruses (MMTVs): (i) cells infected with type-D retroviruses exhibit intracytoplasmic A particles, (ii) virions bud with a complete nucleoid formed, and (iii) virions contain a RNA directed DNA polymerase with a Mg^{2+} cation preference over Mn^{2+}.

The first type-D isolate was the Mason-Pfizer virus (MPV). This agent was initially observed by electron microscopy in a spontaneous carcinoma in the breast of a female rhesus monkey. Other isolates, extremely similar or identical to MPV, have since been obtained from rhesus lactating mammary glands and rhesus placenta cultures. A virus morphologically indistinguishable from MPV has recently been isolated from the spectacled langur (Presbytis obscuris). This virus, designated PO-1-Lu or langur virus (LV), was isolated by co-cultivation of langur lung cells with bat and human cells. Both the rhesus and langur are Old World monkeys. The squirrel monkey (Saimiri sciureus) is a New World species. A type-D retrovirus, similar in morphology to MPV, was isolated from several squirrel monkey tissues by the co-cultivation of those tissues with cells of a variety of species. This has since been designated squirrel monkey retrovirus (SMRV).

There have also been numerous reports (at least fourteen) of type-D isolates from a variety of human cells. These cell lines include HeLa clones and AO cells; some of the lines (including AO cells), however, have been shown to contain HeLa markers. We have previously reported no detectable differences in nucleotide sequence homology between MPV and RNA isolated from HeLa and AO cells producing particles similar to MPV. No MPV related sequences were found, furthermore, in the RNA or in the DNA of two clones of HeLa cells obtained from the American Type Culture Collection. These studies provide strong evidence that at least some of the human isolates appear to be the result of viral and/or cellular contamination. These studies, however, do not mean that any of the other

MPV related isolates from human cells are indeed not bona fide human isolates; further studies are clearly required to clarify this point.

MAJOR FINDINGS:

Iodinated MPV 60-70S RNA has been used in molecular hybridization experiments to determine the distribution of MPV specific proviral sequences in the DNAs of primates. Approximately 20% of the MPV genome was shown to be present as endogenous provirus in rhesus monkeys. No MPV specific proviral sequences were detected in the DNAs of apparently normal tissues of various species of New World monkeys, apes, and humans. The part of the MPV genome that is endogenous to rhesus monkeys has also been shown to be endogenous to the other species of Old World monkeys examined; baboon, African green, and patas. Competitive hybridization experiments showed no homology between MPV 60-70S RNAs and the genomes of of numerous type-C and type-B retroviruses.

The use of relaxed hybridization conditions revealed a second class of "MPV-related" sequences in the DNAs of primates. These "MPV-related" sequences, which are similar to an additional 40% of the MPV genome, were detected when the temperature of hybridization was reduced to 54°C and when the resulting ^{125}I-labeled RNA-DNA duplexes were scored for RNase resistance in 8X SSC. These sequences were found in the DNAs of all Old World monkeys examined; the Tm values of the hybrid duplexes were approximately 6-7°C lower than those of the hybrid duplexes formed using standard hybridization conditions. Gene sequences related to the langur virus have been found in multiple copies of langur cellular DNA and partially homologous sequences were also found in the DNA of related Old World monkey species. Therefore, one explanation of these findings is that MPV, now infectious among primates, may have been derived from the endogenous virus of the langur or a related primate. We have also recently shown that several full term rhesus placentas examined contain additional MPV proviral sequences in their DNA. Competitive molecular hybridization experiments demonstrated that some of these placentas also contain RNA complementary to the entire MPV 60-70S RNA genome. These results provide further evidence that MPV is being transmitted via a nongerm line mechanism in the rhesus population and demonstrate the placenta as a good source for the identification of retrovirus transcriptional products and proviral DNA.

We have also demonstrated that squirrel monkey retrovirus, SMRV is an endogenous virus of squirrel monkeys. ^{3}H-labeled 60-70S SMRV RNA, isolated from virus grown in canine cells, hybridized to the same extent and to the same C_0t 1/2 value (low repetitive range) to the DNA of all tissues of all squirrel monkeys tested. No SMRV proviral sequences were detected, however, in tissues from a variety of other New World monkeys, Old World monkeys, or apes. Competitive molecular hybridization studies also revealed no detectable sequence homology between the RNA genomes of SMRV and MPV.

The polypeptide composition of MPV has been determined using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Six major polypeptides of molecular weights of 68,000, 27,000, 20,000, 14,000, 12,000, and 10,000 were resolved regardless of the cell type in which the virus was grown. Gp68 (68,000) was shown to be the major envelope glycoprotein, and gp20 a minor glycoprotein of the virion, regardless of the cell type of origin of the virus. The structural proteins of LV have also been analyzed and appear to be extremely similar to those of MPV. The structural proteins of SMRV, however, are quite distinct from those of MPV and LV. When analyzed by tube SDS gel electrophoresis, the major virus associated polypeptides display molecular weights of 75,000, 36,000, 20,000 and 12,000. The 75,000 protein was shown to be a glycoprotein by ^{3}H-glucosamine labeling and by periodic acid Schiff staining.

Radioimmunoassays (RIAs) for the p27 major structural protein of MPV revealed no immunologic cross reactivity with 13 different type-B and type-C retroviruses. Homologous RIAs for the p27 of MPV or the p27 of LV cannot distinguish these two viruses. LV and MPV can be differentiated, however, by using either of the RIAs developed for their 10-12,000d proteins. The major structural protein of SMRV was shown to be more immunoreactive with LV than it was with MPV. Interspecies competition radioimmunoassays have recently been developed in which the type-D retroviruses from three primate species compete. One such assay utilized the major structural protein (p36) of SMRV and antisera directed against the major structural protein (p27) of MPV. Addition of the langur monkey virus also resulted in complete blocking. No blocking in the assay was observed with type-C baboon viruses, woolly monkey virus, and gibbon virus and various other type-C and type-B viruses. An interspecies assay has thus been developed that recognizes the type-D retroviruses from both Old World monkey (rhesus and langur) and New World monkey (squirrel) species.

Competitive radioimmunoassays for the internal polypeptides p27 and p10-12s of MPV and LV have recently been used to demonstrate that the type-D retroviral p10-12s exhibit group specific determinants, while the p27 RIAs detect primarily interspecies determinants. Type-D retroviruses isolated from various primate cells and human tumor cells are now being examined in these assays. Virus stocks are normalized for p27 content and then examined in RIAs for MPV and LV p10-12's. No detectable differences were found between MPVs derived from two different cell lines and X-381, a type-D retrovirus isolated from a rhesus mammary gland; nor were there significant differences between MPV and the type-D retroviruses from the human cell lines HeLa and AO. The type-D retroviruses from T9 and J96 human tumor cell lines, however, competed partially in the p10-12 RIA for MPV, and were also shown to differ significantly from the p10-12 of LV. These assays thus demonstrate that the type-D retroviruses isolated from some human cells differ significantly from the prototype type-D retroviruses isolated from nonhuman primates.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROPOSED COURSE: Type-D retroviruses have now been isolated from the three different nonhuman primate species and a variety of human cell lines. Any RNA virus containing an RNA-directed DNA polymerase should be considered as a candidate transforming virus. The similarity of this group of viruses with type-B MMTVs and the fact that MPV and X-381 viruses were isolated from a rhesus mammary tumor and lactating mammary gland, respectively, invites studies to determine if a relationship exists between this group of viruses and mammary carcinoma. The various interspecies RIAs for type-D retroviral gene products will be employed toward this end.

PUBLICATIONS:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04896-08-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Analysis of gene controlled events in neoplastic transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Immunology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2.0	2.0

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sexual and parasexual genetic analysis has permitted the detection, characterization and genetic localization of a number of mammalian cellular genes which participate in endogenous type C virus expression. Six classes of genes are under study: (1) endogenous cellular DNA sequences homologous to type C viral RNA; (2) integration sites of retroviruses in mammalian chromosomes; (3) viral cell surface receptors; (4) restriction genes which delimit viral replication; (5) enzyme structural genes; (6) cell surface antigens including MHC. Examples of each of these gene classes have been detected and mapped in human and feline model systems. The allozyme genetic signature as a definitive characteristic of cultured cells has been applied to monitors of cell contamination, quality control of in vitro transformation and identification of tissue origin in human embryonic development. Allozyme typing and karyologic analysis has demonstrated the triploid and androgenetic origin of partial and complete hydatidiform moles respectively. Population genetic analysis of human feline, and murine populations has revealed evolutionary conservation of linkage associations and the characters of genetic variance.

Project Description

Objectives:

1. The construction of a biochemical genetic map of domestic cat (Felis catus) with emphasis on genes which relate to the extraordinary viral etiology of leukemia and lymphoma in the species. The specific classes of genes under study fall into six general groups, four of which relate to type C RNA retrovirus and replication. The genes include: (1) endogenous cellular DNA sequences homologous to cDNA radioactive probes transcribed from retroviral genomic RNA; (2) chromosomal integration sites for exogenous retroviral insertion and persistence; (3) receptors on cell membranes which interact with virus glycoproteins to determine cell species compatibility and viral host range; (4) restriction genes which delimit virus replication in various animal species; (5) cellular enzyme structural genes; (6) cell surface antigens including antigens homologous to the MHC of other mammalian species.

2. The combined application of somatic cell genetics and electrophoretic resolution of cellular DNA sequences following digestion with specific restriction endonucleases to the biology of retroviral integration and excision in human and feline cells.

3. Genetic analysis of cooperative and sequential gene action in the neoplastic processes and in virogene expression. This consideration involves application of the principles and techniques of microbial genetics to cultured mammalian cells.

4. The development of new approaches to the understanding of genetic control of carcinogenesis. These considerations involve the study of genetic targets (cellular genes) of carcinogenesis, and the analysis of population genetic structure of natural populations subject to specific types of virus associated cancer.

5. Use of inherent genetic variation in human and mouse cells to provide genetic signatures or markers for cells studied in cell culture laboratories. By using allelic isozyme (allozyme) variation in different isolates of human or mouse cells, an allozyme genetic signature of cells can be derived for cell contamination monitors and for approaches to the genetics of development.

Methods Employed:

The following techniques are employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3)) cytological chromosome banding procedures (G and Q) for specific chromosome identification; (4) virological procedures including radioimmune assay, reverse transcriptase and viral cloning; (5) immunological assays including RIA, cytotoxicity, fluorescent antibody procedures, and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques including

^{3}H-cDNA transcription in vitro, solution hybridization, HAP chromatography, visualization of restricted DNA by the techniques of Southern following agarose electrophoresis.

Major Findings:

A preliminary requirement for this effort is the availability of a substantial biochemical genetic map of the cat.

Briefly, some 36 different cell hybridization crosses between fresh diploid cat tissue or heteroploid cell lines with mutant rodent cell lines have generated over 700 primary hybrids and over 200 secondary and tertiary subclones. Over 40 gene-enzyme systems were followed in various crosses and a number of segregationally autonomous linkage groups (17) have been derived from synteny (concordance) data in diploid and heteroploid dervied hybrids. The haploid number of cats is 19. Seven of the linkage groups contain loci that are also syntenic in man indicating conservation of linkage relationships of large blocks of genes in mammalian chromosome evolution. The linkage groups have been assigned to specific cat chromosomes using trypsin-G-banded karyotypes of hybrids in more than half of the 19 cat chromosomes. The map is not yet complete but the nucleus of biochemical markers is an important prerequisite for somatic cell genetic analysis of virogenes. We have discovered twelve (12) polymorphic biochemical loci in a population survey of feral cats collected in eastern United States. These markers are in addition to the fifteen unmapped morphological loci previously defined and fixed in various combinations in the 100-odd breeds of cats presently registered with the Cat Fanciers Association, Inc. We have also identified over twenty biochemical genetic variants which segregate in interspecies sexual crosses between Felis catus and Felis bengalensis. The sum of these biochemical genetic characteristic render the domestic cat now a reasonable model for genetic analysis.

Several feline genes which participate in type C virus replication have been under investigation. The endogenous RD114 and FeLV sequences are distributed on multiple autosomes in cats and segregate independently of each other in rodent x cat hybrids which lose feline chromosomes. In addition the FeSV specific endogenous sequences (not present in FeLV genomes) are observed in certain segregant clones. Pilot experiments detecting FeLV integration and FOCMA expression indicate that these functions distribute as single loci in segregant clones. An X-linked restriction gene, Bvr-1 (BALB virus restriction), was discovered and characterized as a late acting feline gene which restricts release of murine B-tropic virus and induction of murine X-tropic endogenous virus in cat x mouse hybrids. Bvr-1 is trans-dominant in its action, pleiotropic in viral targets (N-tropic virus is not susceptible) and appears to modify post transcriptional processing of viral particles. A possible human locus homologous to Bvr-1 is suggested in (MuLV) mouse x human hybrid cells.

A human locus, Bevi (for baboon endogenous virus integration), has been mapped to human chromosome six and is required for successful infection of rodent x human hybrids with baboon endogenous virus. Molecular hybridization experiments with cDNA probes prepared with baboon virus to cellular DNA of $Bevi^+$ and $Bevi^-$ hybrids of infected human x hamster crosses have demonstrated that Bevi is a high affinity integration for baboon endogenous virus in the human genome. The Bevi locus represents the first genetic localization of a human gene function which is involved in RNA tumor virus replications.

The Allozyme genetic signature of human cells (composite allozyme phenotype at eight gene-enzyme systems that are polymorphic in human populations) has found a variety of applications in human cell biology and genetics. Over thirty human cell contaminants have been identified from materials submitted for signature analyses. Signatures of amniotic cells have been used to confirm fetal versus maternal cells for purposes of genetic counseling. The triploid and androgenetic origin of partial and complete hydatidiform moles respectively has been achieved. These carcinomas develop in pregnant women and represent abnormal fetal development. Ten isozyme systems have been resolved in cultured mycoplasmas and in mycoplasma infected cells. The systems are useful in identification and discrimination of different mycoplasmales as well as indicators of mycoplasma contamination of cultured cells.

The population genetic structure of two classes of mouse populations important in cancer research was estimated. These include the out-bred Swiss mouse colonies of NIH, Charles River, and Eppley Institute, and the wild viremic California mouse population at Lake Casitas, and non-viremic population at Bouquet Canyon. The out-bred Swiss mice were found to contain nearly the same amount of genic variation as wild populations (sample 46 loci) despite laboratory propagation for over 50 years. Thus, they are as advertised genetically outbred. The viremic and non-viremic California populations had similar amounts of genic variation and could not be distinguished in their genetic structure. Evidence for linkage disequilibrium or selective factors in the viremic population compared to the control were not apparent.

The Lake Casitas (LC) mouse population is also a rich source of polymorphisms of viral restriction genes. Akvr-1 is a gene which restricts the AKR viremia and virus associated leukemia in LC X AKR hybrid mice. Akvr-1 is a new restriction gene in mouse distinct from Fv-1, Fv-2 and other retroviral restriction genes. Indirect evidence suggests the participation of two additional polymorphic restriction loci in the LC population: (1) a gene which confers resistance lymphoma in mice infected with the exogenous LC amphotopic virus, the etiological agent of lymphoma; and (2) a gene which confers resistance to amphotropic virus infection. Sexual genetic analysis of these three genes and related murine restriction genes is in progress.

Significance to Biomedical Research and the Program of the Institute:

The analysis of the metabolism of leukosis virus infection and its relationship to antigen assembly provides important information about the course of cell modification as the result of tumor virus infection and expression. The understanding of the virus and host genetic machinery active in a malignant cell is necessary for any meaningful attempt to correct or destroy cancerous tissue.

The parasexual genetic analysis of gene action following neoplastic transformation is an important approach to the understanding of genetic regulation of cell division in normal and malignant mammalian cells.

Publications:

O'Brien, S.J. and Rice, M.C.: Genetic aspects of carcinogenesis and carcinogen testing in outbred Swiss mice. J. Toxicol. and Envir. Health 5:69-81, 1979.

Noguchi, P., Wallace, R., Johnson, J., Early, E.M., O'Brien, S.J., Ferrone, S., Pelegrino, M.A., Milstein, J., Needs, C., Browne, W. and Pettricianni, J.: Characterization of WIDR: a human color carcinoma cell line. In Vitro 15:401-408, 1979.

Surti, U., Szulman, A.E. and O'Brien, S.J.: Complete hydatidiform mole with 46,XY karyotype of paternal origin. Human Gen. 51:153-155, 1979.

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O'Brien, S.J., Shannon, J.E. and Gail, M.H.: A molecular approach to the identification and individualization of human and animal cells in culture: isozyme and allozyme genetic signatures. In Vitro 16:119-135, 1980.

Pearson, P.L., Roderich, T.H., Davisson, M.T., Garver, J.J., Warburton, D., Lalley, P.A. and O'Brien, S.J.: Comparative Gene Mapping: Committee Report. Cytogen. Cell Genet. 25:82-95, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-04948-05-LCMB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Genetic Studies of Mammalian Type-C Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH Carcinogenesis Intramural Program
Laboratory of Cellular and Molecular Biology

SECTION Molecular Biology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to 1) develop radioimmunologic techniques to search for retrovirus expression in cells and tumors of human origin, 2) isolate recombinants between mouse leukemia viruses to provide genetic evidence for viral genes involved in leukemogenesis, 3) establish the genetic map of mammalian type-C viruses, 4) identify and characterize the transforming proteins of murine and feline sarcoma viruses and 5) determine the mechanisms of sarcoma virus-induced cell transformation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1. To develop sensitive radioimmunoassays for the major structural proteins of mammalian oncoviruses to search for the presence of antibodies against oncoviruses in humans as well as for oncoviral protein expression in normal and tumored human cells.

2. To characterize the oncogenic properties of recombinants between mouse type-C viruses to determine the existence of viral genes directly involved in leukemogenesis.

3. To establish the genetic map of mammalian type-C viruses by immunologic and biochemical characterization of recombinant viruses.

4. To identify and characterize the translational products of murine and feline sarcoma viruses.

5. To study the mechanisms of sarcoma virus-induced oncogenesis.

Major Findings:

1. We have developed radioimmunologic techniques that have made it possible to detect cross-reactivities among the major structural proteins of prototype viruses representing each of the three major Oncovirinae genera. Two different sets of antigenic determinants were demonstrated. One was shared by type-B and type-D viruses, whereas the other was found to be common to type-C and type-D viruses. These findings provide experimental evidence supporting the concept of an evolutionary relationship among the three major oncovirus genera.

2. The relationships between OMC-1, an endogenous oncovirus of owl monkey, and representatives of the three oncoviral genera was investigated by radioimmunological techniques. The major structural protein of OMC-1 was shown to share antigenic determinants with the corresponding proteins of certain type-C viruses of rodent, feline, and cervine origin. Furthermore, a close antigenic relationship was established between the major structural proteins of OMC-1, an endogenous virus of deer (deer kidney virus), and avian reticuloendotheliosis viruses. These findings establish OMC-1 and deer kidney virus in the evolutionary lineage that may have led to the generation of avian reticuloendotheliosis virus, a group of oncogenic viruses capable of crossing the interclass barrier between mammals and birds.

3. We have investigated the specificity of certain reports claiming presence in humans of antibodies directed against oncoviral glycoproteins. We observed that such antibodies can be detected under

certain assay conditions. Substances as diverse as normal components of serum, extracts of bacteria, and even nonprotein molecules such as glycogen shared the oncoviral glycoprotein determinants recognized by normal human sera. Moreover, immunoprecipitation of a given viral glycoprotein by human sera was entirely dependent upon the cell in which the virus was grown. These findings not only demonstrate that the antibodies were directed against cellular rather than the virus-coded antigenic determinants but also exclude the possibility that this immune response was elicited as a consequence of oncovirus exposure.

4. We have generated in tissue culture recombinants between a potent murine leukemia virus, Rauscher-MuLV, and an endogenous xenotropic mouse type-C virus, BALB:virus-2, without known malignant potential. We used a double selection system devised to select against the temperature-sensitive (ts) lesion associated with a mutant of Rauscher-MuLV and the xenotropic host range of BALB:virus-2. Recombinant viruses, obtained at frequencies ranging from 0.01 to 0.1%, were identified on the basis of the type specific antigenic determinants in the translational products. By this approach, the partial genetic maps of a large number of recombinants were obtained. These viruses should be useful in defining those regions of the viral genome involved in leukemogenesis.

5. We have determined that the ST-, GA- and SM-strains of feline sarcoma virus (FeSV) code for high-molecular weight polyproteins that contain varying amounts of the amino-terminal region of the FeLV gag gene-coded precursor proteins and a polypeptide(s) of an as yet undetermined nature. The SM-FeSV primary translational product is a 180,000 dalton polyprotein, which contains a 120,000 dalton FeSV-specific polypeptide. The GA- and ST-FeSV genomes code for polyproteins of 95,000 and 85,000 daltons, respectively, which in addition to the amino-terminal moiety (p15-p12 and a portion of p30) of the FeLV gag gene-coded precursor protein also contain FeSV-specific polypeptides. Immunological and biochemical analysis of each of the above FeSV translational products revealed that the sarcoma-specific regions of the GA- and ST-FeSV polyproteins are antigenically cross-reactive and exhibit common methionine-containing peptides. These findings favor the concept that these sarcoma-specific polypeptides are coded for by the similar subsets of cellular sequences incorporated into the GA- and ST-FeSV genomes during the generation of these transforming agents.

Significance to Biomedical Research and the Program of the Institute:

The applicability of biochemical radioimmunological techniques to the identification and characterization of proteins coded for by the genome of tumor viruses should be of great value in the search for the primary cause of cellular transformation by these viruses.

Proposed Course:

1. To continue developing highly sensitive radioimmunologic techniques capable of detecting oncoviral antigens in tumors from species from which oncoviruses have not yet been isolated. These radioimmunologic techniques will be useful in identifying and characterizing new oncoviral isolates.

2. To establish genetic markers in order to identify the viral genes involved in leukemogenesis.

3. To determine the molecular mechanisms by which the gene products of murine and feline sarcoma viruses cause malignant transformation.

Publications:

Barbacid, M., Long, L.K., and Aaronson, S.A.: Major structural proteins type-B, type-C and type-D oncoviruses share interspecies antigenic determinants. Proc. Natl. Acad. Sci. USA 76: 72-76, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-04996-03-LVC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunologic Studies of Mammary Carcinomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Tumor Virus Detection Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to determine the spectrum of type-B retroviral translational products that exists in various murine tissues and to employ this knowledge to elucidate the role of the host's immune response to these products in mammary tumorigenesis. Major accomplishments during the past year have included: (a) the development of the first sensitive and specific competition radioimmunoassays (RIAs) for the major envelope glycoproteins (gp52 and gp36) and major core protein (p28) of MMTVs. These assays have been used to analyze the antigenic diversities and similarities occurring in MMTVs from different high and low oncogenic MMTVs obtained from the same mouse; (b) the determination that the previously considered model of an "MMTV-free" mouse, i.e, BALB/c, demonstrates non-coordinate expression of specific MMTV-translation products in both preneoplastic and neoplastic mammary tissues; (c) the generation of a library of monoclonal antibodies to specific MMTV antigenic determinants. These have been used to define the broad diversity that exists among MMTVs and to further define the host's humoral immune response to spontaneous mammary carcinoma.

Project Description

OBJECTIVES: To determine the spectrum of type-B retroviral translational products that exists in various murine tissues and to employ this knowledge to elucidate the role of the host's immune response to these products in mammary tumorigenesis. Specific aims include:

(a) The development of sensitive and specific competition radioimmunoassays (RIAs) for the major envelope glycoproteins (gp52 and gp36) and major core protein (p28) of MMTVs. These assays will be used to determine the degree of diversity that exists among MMTVs derived from various high and low mammary cancer incidence mouse strains as well as to differentiate between high and low oncogenic MMTVs obtained from the same mouse.

(b) Examine the "MMTV-free" BALB/c mouse to determine the relationship, if any, between the expression of MMTV-translational products and spontaneous mammary cancer.

(c) Generate monoclonal antibodies to specific MMTV antigenic determinants to be employed to further define the host's humoral immune response in spontaneous mammary carcinoma.

BACKGROUND AND RATIONALE: Various strains of mice differ in their incidence of mammary tumors, latent period to tumor formation, and type of tumor formed. To assess the role of a viral involvement in these parameters, it is important to distinguish between virus-directed and host-controlled activities. Previous reports have indicated some differences in biological activities of different mouse mammary tumor virus (MMTV) isolates. Recent studies in our lab have further shown that some MMTVs may differ substantially both in the sequences of their RNA genomes and in the mechanism by which they are transmitted. Previous reports utilizing RIAs for the external glycoprotein gp52, however, have not detected differences among various MMTVs; i.e., only group-specific reactivities have been reported. Recent studies have delineated the polypeptide structure of the MMTV virion. The major external glycoprotein has a molecular weight of 52,000 (gp52). Other major virion proteins include a glycoprotein of 36,000 daltons and polypeptides of 28,000, 14,000, and 10,000 daltons. A central issue is the determination of the diversity of mammary tumor viruses (and their association with mammary tumors) that exist in different mouse strains or within a given mouse.

MAJOR FINDINGS:

Several investigators have previously shown that the gp52 of MMTVs share group-specific antigenic determinants. We have conducted experiments that demonstrate type-specific antigenic determinants also exist on the gp52 molecule. The highly oncogenic MMTV(RIII) and MMTV(C3H) were clearly differentiated when assayed in a competitive radioimmunoassay (RIA) using anti-MMTV(C3H) to bind ^{125}I-labeled MMTV(C3H) virions. In this system, increasing amounts of MMTV(RIII) competitor presented a shallower slope

than that given by the isologous MMTV(C3H). In a "group-specific" assay using anti-MMTV(C3H) vs. ^{125}I-MMTV(RIII), both viruses competed identically, i.e., at comparable inputs and with the same slope. Additional type-specific reactivities among the various MMTVs were also shown to exist. These include differences between the exogenous high oncogenic MMTV(C3H) and the endogenous low oncogenic MMTV of C3H obtained from C3HfC57BL mice. The type and group specificities of MMTVs grown in feline cells were indistinguishable from the reactivities observed with murine grown MMTVs, thus providing strong evidence that the MMTV gp52 antigens are viral coded.

We have recently developed the first competition RIA for the major internal protein (p28) of MMTV. When this assay was conducted with high antibody dilutions for maximum sensitivity, no differences were observed among MMTVs from RIII, GR, C3H, or C5HfC57BL mice or with mammary tumor extracts from those mice. To demonstrate type-specific reactivities associated with the MMTV p28 polypeptides, assay conditions of low antibody dilutions were used. Thus, the MMTV p28 appears to contain both indistinguishable, i.e., group-specific, antigenic determinants, as well as distinguishable, i.e., type-specific, antigenic determinants. Using the combination of gp52 and p28 RIAs, levels of MMTV gene expression can now be analyzed in terms of both viral glycoproteins and nonglycoproteins. This type of comparison is important in light of our recent evidence of noncoordinate polypeptide chain initiation of glycosylated vs. nonglycosylated MMTV proteins in infected cells.

The virion envelope of the mouse mammary tumor virus (MMTV) is composed of two major glycoproteins with estimated molecular weights of 52,000 (gp52) and 36,000 (gp36). The external location of these two virion structural components and their association with the morphologically distinct surface projections of the MMTV B-particles have been previously documented. It has also been established that these two glycoproteins are cleaved from a common precursor molecule; recent tryptic peptide map analyses have demonstrated that gp36 and gp52 each have a distinct primary amino acid sequence. Several earlier studies have dealt with the potential significance of MMTV gp52 in mammary tumorigenesis; however, analogous studies with MMTV gp36 have not been reported. This may be due in part to the difficulty in purifying gp36. A recent report on the relative hydrophobicity of the MMTV structural proteins described a method whereby appreciable quantities of gp36 can be purified. Using this method, followed by an additional gel filtration step, we have purified gp36 from MMTV virions and have developed radioimmunoassays for the detection of MMTV gp36-related antigens.

The gp36 molecules of MMTVs have now been demonstrated to have multiple antigenic determinants, i.e., group-specific antigens common to all strains of MMTV tested, and type-specific antigens by which MMTVs can be differentiated from one another. The sensitivity of the gp36 RIA is approximately the same as that of the gp52 RIA; both assays can be used to

detect as little as 0.2 ng of MMTV-related antigen. Type-C and type-D retroviral proteins, and normal cellular antigens, and serum proteins do not compete in any of the above RIAs.

Noncoordinate Expression of MMTV Gene Products in "MMTV-free" Mice.

Numerous mouse strains are used as animal models for mammary tumorigenesis. Strains that have a low spontaneous mammary tumor incidence, such as BALB/c, are particularly interesting since they are highly susceptible to mammary tumor induction by a variety of hormonal and chemical agents. Previous studies have demonstrated that both D2 preneoplastic hyperplastic alveolar nodule (HAN) outgrowth lines and mammary tumors derived from them in BALB/c mice contain significant levels of MMTV specific RNA. However, mammary tumors arising from the D2 HAN line have been routinely negative for MMTV B-particles by electron microscopy. Highly concentrated supernatant fluids of primary cultures of D2 mammary tumor cells also showed no evidence of MMTV virions by electron microscopy or by radioactive tracer studies.

We are now involved in studies to examine "virus-free" preneoplastic nodules and tumors for the expression of MMTV antigens employing the group-specific RIAs for MMTV p28, gp52, and gp36 described above. These studies are providing evidence that high levels of MMTV p28 may be expressed in the absence of MMTV gp52 and gp36. Extracts of spontaneous mammary tumors of MMTV positive C3H mice demonstrate significant competition in all three RIAs. However, the addition of cell free extracts of D2 mammary tumors to each RIA showed the extracts to contain p28 antigen without MMTV gp52 or MMTV gp36. In several samples, less than 0.000003% of the total protein in the D2 mammary tumor extract was related to MMTV gp52, while approximately 0.2% of the total protein in the same sample was related to p28. Several different transplant generations of the D2 mammary tumor in BALB/c mice have been assayed for MMTV antigens and in all the tumor extracts examined, noncoordinate expression of MMTV p28 versus MMTV gp52 was found.

Studies were undertaken to determine if this phenomenon of noncoordinate expression was observed in other "MMTV-free" murine mammary tissues, or if it was a unique property of BALB/c D2 mammary tumors. Extracts of primary mammary adenocarcinomas arising in BALB/c mice after hormonal stimulation competed completely in the MMTV p28 RIA; the amount of p28 present in these tumors was the same as that found in a spontaneous tumor arising in MMTV infected BALB/cfC3H mice. In contrast to the BALB/cfC3H tumors which competed completely in both of the glycoprotein RIAs, the BALB/c tumors showed no significant competition in either the gp52 or gp36 RIAs. Mammary adenocanthomas also arise after hormonal stimulation in some BALB/c mice. These adenocanthomas, however, did not appear to express any of the three MMTV antigens being assayed. Normal mid-pregnant mammary glands and benign dysplasias from BALB/c mice also failed to show expression of MMTV antigens.

Noncoordinate expression of MMTV antigens was not restricted to the BALB/c strain. Swiss albino mice carry a milk borne MMTV. This MMTV can be removed by foster nursing newborns on MMTV negative C57BL mice. Normal mammary glands from both the "MMTV positive" Swiss mice and the "MMTV negative", foster nursed, Swiss mice were assayed for MMTV antigens. Whereas the MMTV positive mammary glands showed significant quantities of MMTV p28, gp52, and gp36, mammary glands from foster nursed Swiss mice were found to contain high levels of MMTV p28, but no MMTV gp52 and little, if any, MMTV gp36. The biological significance of noncoordinate expression of MMTV antigens in murine mammary tissues is currently under investigation. It is noteworthy, however, that this phenomenon exists in both primary and transplanted mammary tumors, and in preneoplastic mammary tissues of several colonies of BALB/c and in normal mammary tissue of Swiss albino mice. It appears that noncoordinate expression of MMTV antigens may be a more common phenomenon in murine mammary tissues than has previously been conceived.

Monoclonal Antibodies to MMTV Antigenic Determinants.

Monoclonal antibodies, synthesized in-vitro following the fusion of lymphocytes from immunized mice or rats with nonproducer murine myeloma cells, have proven quite useful in the detection and characterization of tumor associated antigens (both viral and non-viral) in several animal and human tumor systems. Employing this now classical approach, we have generated monoclonal antibodies to numerous distinct antigenic determinants that reside on the 52,000d and 36,000d glycoproteins, or the 28,000d major internal protein of mouse mammary tumor viruses (MMTVs). We have utilized these immunoglobulins to: (a) further define type, group, and interspecies antigenic determinants among MMTVs, and (b) elucidate how these distinct antigenic determinants present themselves in spontaneous mammary tumors of several species. Employing the immunoperoxidase technique, we have examined different regions of a single murine mammary tumor, different mammary tumors from a single inbred mouse strain, and mammary tumors from several rodent species. An outstanding feature of all these studies was the heterogeneity in expression of distinct antigenic determinants that exists not only between different mammary neoplasms, but also within individual regions of a given tumor.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROPOSED COURSE: These studies have for the first time demonstrated the broad diversity in translational products that exists in the family of viruses known as "MMTV". Until quite recently the argument was still raging as to whether "MMTV" was actually more than one virus. The RIAs developed in our lab for the gp52, gp36 and p28 proteins clearly demonstrate that MMTVs form a multigene family, the full extent of diversity of which is still unknown. Our studies involving the generation of monoclonal antibodies will further refine the classification and distinction between MMTVs and should lead to a knowledgeable dissection of the host's humoral immune response to distinct MMTV coded translational products. The studies revealing

noncoordinate expression of MMTV gene products, in strains of mice previously thought to be the "classical model" for a virus free system, are now leading to studies on the influence of hormones and chemicals on the expression of specific viral translational products, rather than on the synthesis of whole virions as previously conceived.

PUBLICATIONS:

Teramoto, Y. A. and Schlom, J.: Radioimmunoassays for the 36,000 dalton glycoprotein of murine mammary tumor viruses demonstrate type, group, and interspecies determinants. J. Virol. 31:334-340, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05070-02-LVC

PERIOD COVERED

September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Temperature Sensitive Mutants of Moloney Murine Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	P. J. Fischinger, Head, Virus Control Section	LVC, NCI
	W. G. Robey	LMV, NCI
	G. F. VandeWoude, Head, Virus Tumor Biochemistry Section	LMV, NCI
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COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

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NCI, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Summary

Moloney MSV temperature sensitive transformation mutants have been isolated which exhibit coordinate temperature sensitive expression of several transformation parameters. Genetic and biochemical studies of one of these isolates, ts110, and several spontaneous wild type revertants are in progress. The ts MSV genome has been transferred to normal mouse cells by DNA transfection using high molecular weight DNA from temperature sensitive transformed rat cells. MSV pseudotypes rescued from ts110 transformed rat cells contain a heat labile low molecular weight protein kinase. This represents the only instance reported to date where a murine sarcoma virus transformation defect can be correlated with a specific enzymatic defect.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses and the function and cellular targets of specific gene products of MSV in this process.

Methods Employed:

Focus and agar colony formation by MSV on various cell lines in the presence and absence of replicating helper viruses; DNA transfection using cloned MSV sequences and whole cell DNA from MSV transformed cells; recombinant DNA cloning of integrated MSV sequences from transformed cells; restriction endonuclease mapping and heteroduplex analysis of cloned sequences.

Major Findings:

TS110 is a MSV transformation mutant which maintains a stable ts phenotype under continued passage at the permissive temperature (34^{o}). This line exhibits extensive morphological heterogenecity and contains many cells which have a nearly normal (flat) morphology at the permissive temperature. However, these morphologically heterogeneous cultures show no change in their ability to express another transformation parameter, so growth in agar suspension. Rescue of MSV with a variety of helper viruses (MoMLV, BMux, HIX, SSAV) results in the appearance of between 10% and 50% phenotypically wild type MSV. Transfer of the genome via DNA transfection (in the absence of replicating helper) appears to show a much lower frequency of reversion. Preliminary results indicate we have cloned the ts110 MSV genome from transformed rat cells.

A low molecular protein kinase ($\sim$14,000 daltons) isolated from MLV-rescued viral supernates and purified by actin affinity chromatography is 10-fold more temperature sensitive than that present in the wild type (parent) strain of MSV rescued from rat cells using the same helper. Preliminary evidence indicates that the kinase isolated from one spontaneous revertant remains ts although the level of kinase appears higher than in the ts parent. Biological analysis indicates virus rescued from this revertant is phenotypically wild type.

Significance to Biomedical Research and the Program of the Institute:

Conditional mutants have been extremely useful in elucidating biochemical pathways in a number of cell and viral systems. The MSV mutants described here represent the first isolation of conditionally defective varients of a mouse-derived sarcoma virus. Their isolation has allowed the identification of a ts enzymatic function which can be correlated with the trans-

formed phenotype. Further analysis of these and other mutants should determine whether this activity plays a direct role in the primary, virus induced transformation of normal cells. The availability of cloned MSV carrying a defective transformation gene should aid in the identification of specific regions of the genome involved in the biological expression of the transformation function, and should lead to the identification of the defect at a nucleotide level.

Proposed Course:

Using DNA transfection techniqes, we will attempt to complement the ts transformed NIH3T3 cells derived by transfection with cloned whole and partial genomes of wt MSV. We will also characterize the cloned ts110 biochemically by restriction mapping and heteroduplex analysis, and biologically with DNA transfection analysis. We intend to characterize the biological properties of several revertants which arose spontaneously or as a result of MLV rescue and analyze the viral RNA and subgenomic src containing messenger RNA to determine if overproduction of viral products, mutation or recombination is responsible for the reversion observed.

Publications:

Blair, D. G., Hull, M. A., and Finch, E. A. The isolation and preliminary characterization of temperature sensitive transformation mutants of Moloney sarcoma virus. Virology 95: 303-316, 1979.

Sen, A., Todaro, G. J., Blair, D. G., and Robey, W. G.: Thermolabile protein kinase molecules in a temperature sensitive murine sarcoma virus pseudotype. Proc. Natl. Acad. Sci. USA 76: 3617-3621.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05071-02-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Experimental Basis for a Molecular Approach to Cancer Prophylaxis and Therapy for Some Types of Human Neoplasia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. A. Phillips	Research Microbiologist	LVC	NCI
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COOPERATING UNITS (if any)
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to make possible a molecular approach to cancer prophylaxis and therapy for some types of viral neoplasia, especially human leukemia and cancer of the breast where retrovirus information has been detected. Major findings to date are: (1) Poly(A), poly(C), and poly(G) sequences are present in all retrovirus RNAs examined. (2) Poly(U) sequences are absent in retrovirus RNA. (3) Poly(C) and poly(G) sequences present in retrovirus RNA are transcribed into complementary poly(dG) sequences and poly(dC) sequences respectively in subunit-length cDNA of retroviruses. (4) The 3'-terminal poly(A) sequences are not transcribed into poly(dT) sequences. (5) Little or no poly(C) and poly(G) sequences have been detected in the cellular nucleic acids examined from nonmalignant mammalian and human embryonic cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To establish a sound scientific basis for a molecular approach to cancer prevention and treatment for human cancers in which retrovirus information has been detected. Studies are divided into four phases. (I) The biochemistry and biophysics of retrovirus and cellular nucleic acids: To look for and to characterize, if detected, unique specific nucleotide sequences present in retrovirus nucleic acids which may either be absent or present in insignificant amounts in cellular nucleic acids from nonmalignant mammalian and human cells. (II) The testing of synthetic single-stranded homopolymers, sequences specific drugs, an chemically modified antibiotics *in vitro*: To determine *in vitro* whether these agents can specifically inhibit transcription of integrated retrovirus genes without interfering significantly with the transcription of cellular genes. (III) The testing of synthetic single stranded homopolymer, sequence specific drugs, and chemically modified antibiotics *in vivo*: Animal studies are planned to determine whether the transcription of integrated retrovirus genes can be selectively blocked in mice and other animals that have a high incidence of breast cancer and leukemia by *in situ* hybridization of nuclease protected, single-stranded homopolymers which are complementary to the homopolydeoxyribonucleotide sequences present in integrated and nonintegrated retrovirus DNA and the specific binding or intercalation of sequence specific drugs and chemically modified antibiotics to these like sequences of dCMP and dGMP in the viral DNA. Sufficient testing will be done to acquire investigatory new drug (IND) numbers from the Federal Drug Administration (FDA). (IV) The treatment of human cancers in which retrovirus information has been detected with potential anti-cancer agent(s) found to be most effective in the phase III animal studies.

Methods Employed:

For characterization of the viral nucleic acids and polypeptides from retroviruses, standard biochemical and biophysical techniques (analytical and preparative ultracentrifugation, chromatography, nucleic acid hybridization, isotopic labeling, polyacrylamide gel electrophoresis, peptide and oligonucleotide fingerprinting, electrofocusing, *in vitro* protein synthesis, enzymology, etc.) are being employed. Improved tissue culture techniques are being employed for the growth of retroviruses; infectivity assay of retroviruses; and *in vitro* testing of antibiotics, drugs and homopolymers. Also *in vivo* animal testing of antibiotics, homopolymers, and drugs. Immunodiffusion, radioimmune assays, molecular cloning, agarose gel electrophoresis, southern blot transfer analysis, viral RNA and DNA sequencing, and restriction endonuclease mapping are being used. Amino acid analysis is to be used.

lar nucleic acids. Analysis of homopolyribonucleotide sequences in retrovirus RNA revealed the existence of 3'-terminal poly(A) sequences and internal poly(C) and poly(G) sequences. These sequences have been detected in the RNA of retroviruses from avian to primate isolates. Poly(A) sequences have also been found in all cellular messenger RNA(mRNA), except for histone mRNA, by many investigators. We have shown that comparatively little or no poly(C) and poly(G) sequences exist in poly(A) positive mRNA examined from nonmalignant and embryonic cells. The poly(A) and poly(G) sequences present in the viral RNA are transcribed into poly(dG) and poly(dC) sequences respectively in the viral cDNA, before it becomes integrated into cellular DNA where it apparently remains in an inactive, repressed state until it is activated or derepressed either spontaneously or by chemical or physical carcinogens. The absence of significant amounts of these homopolydeoxyribonucleotide sequences in the cellular DNA from nonmalignant human embryonic cells and their presence in the DNA from retrovirus infected and transformed cells, suggest an experimental basis for a possible molecular approach to cancer prevention and treatment for cancer in which retrovirus information has been detected. Perhaps, the transcription of the integrated viral genes with their unique sequences of dCMP and dGMP could be selectively blocked by either in situ hybridization with complementary synthetic homopolymers to the poly(dC) and poly(dG) sequences in the integrated retrovirus DNA or specifically blocked by sequence specific drugs or chemically modified antibiotics binding or intercalating between the viral specific sequences of dCMP and dGMP. Although human cancers in which retrovirus information has been detected are most probably multifactorial, this unique approach seems worthy of exploring for the prevention primarily and the treatment secondarily of cancer.

Proposed Course:

Phase I: These essential biochemical and biophysical studies on the nucleic acids of mammalian retroviruses and mammalian and human cells will be continued in concert with our phase II, phase III, and phase IV studies. They will be done in order of their complexity and available technology, as well as the availability of adequate professional and technical assistance and funds. Our major research efforts will continue to be directed toward the establishment of a sound scientific basis for a molecular approach to cancer prevention and treatment. The following biochemical and biophysical studies on the nucleic acids of retroviruses and cells, as well as viral glycoproteins, will be performed.

Planned RNA studies: (1) Determination of the location(s) of the poly(C) and poly(G) sequences in the retrovirus RNA by controlled PNPase phosphorolysis of poly(A) positive viral RNA subunits from their 3'-termini, by heteroduplex analysis when it becomes sensitive enough to resolve structures with less than 40 base pairs. (2) Demonstration of the poly(C) and poly(G) sequences in the viral RNA by two-dimensional chromatography in agarose of RNase T1 and RNase C excised tracts. Presently, the like sequences of CMP and GMP in retrovirus RNA have been detected by polynucleotide agarose affin-

Major Findings:

Phase I: (1) The subunit RNA of mammalian sarcoma viruses is approximately 5.0 to 5.8 kilobases in contrast to the larger 5.8 to 10.5 kilobases for the subunit RNA of mammalian leukemia viruses. (2) The 3'-terminus of some viral RNA subunits has poly(A) sequences, or tracts rich in AMP, of variable chain-lengths of 300 nucleotides or less. (3) Poly(A), poly(C), and poly(G) sequences, or tracts rich in AMP, CMP, and GMP respectively, are present in retrovirus RNA from avian to primate isolates. However, poly(U) sequences, or tracts rich in UMP, are absent in retroviruses RNA. (4) Four classes of mammalian virus RNA subunits have been identified according to their homopolyribonucleotide tract compositon and the four subunits assembled into a proposed genomic model for mammalian retroviruses. (5) The poly(C) and poly(G) sequences in the viral RNA are transcribed into poly(dG) sequences, or tracts rich in dGMP, and poly (dC) sequences, or tracts rich in dCMP, in the viral complementary DNA (cDNA). However, the 3'-terminal poly(A) sequences of retrovirus RNA are not transcribed into poly(dT) sequences, or tracts rich in TMP, in the viral cDNA. (6) Comparatively little or no poly(dC) and poly(dG) sequences have been detected in the DNA examined from nonmalignant mammalian and human cells but these sequences are present in the DNA of retrovirus infected and transformed cells, as well as chemically transformed cells, of mammalian and human origins. (7) Genomic and subunit RNA of mammalian leukemia and sarcoma viruses have regions of double-strandedness which are rich in CMP and GMP.

Phase II: We have not made any major findings in these studies, since they were only recently initiated. At this early stage, we have become proficient in biological assays for some of the mammalian retroviruses and in RNA-DNA and DNA-DNA hybridization techniques for the detection of integrated retroviruses DNA in virus infected and transformed mammalian and human cells. However, it has been shown by others (Arya, S.K., Mol. Pharmacol. 13, 585-597, 1977) that poly(I) inhibits the replication of RNA tumor viruses in cultured cells with little or no effect on the growth rate and viability of the cells, and suggest that the inhibition by poly(I) is due, in part, to the inhibition of proviral DNA synthesis. The observed inhibition of retrovirus replication could possibly have been due to *in situ* hybridization of the poly(I) to the poly(dC) sequences present in integrated viral DNA thus selectively blocking the transcription of the integrated viral genes or antiviral action by the possible formation of interferon by the *in situ* formed poly(I):poly(dC) duplexes.

Phases III and IV: These studies have not yet been initiated.

Significance to Biomedical Research and the Program of the Institute:

A molecular approach to cancer prevention and treatment for human cancers in which retrovirus information has been detected, primarily breast cancer and leukemia, has been justified, in part, from our biochemical and biophysical studies on the structure and nucleotide composition of retrovirus and cellu-

ity chromatography, by hybridization or annealing studies, by specific chemical binding studies, and by the ability of oligo(dC), and oligo(dG) to serve as primers in the in vitro synthesis of viral cDNA. (3) Determination of the base composition of excised dsRNA fragments from retrovirus RNA. (4) Determination as to whether the p 12 viral polypeptide binds to the single-stranded or the double-stranded portion of genomic and subunit viral RNA. (5) Reassociation of the four types of subunits into genomic viral RNA in vitro. (6) Characterization of the interstrand and intrastrand double-stranded regions in the genomic and subunit RNA of mammalian retroviruses as to their nucleotide composition, their definitive chain-lengths, and their possible functions. (7) Determination of the role(s) of the poly(A), poly(C), and poly(G) sequences in genomic and subunit viral RNA. (8) Continued examination of the poly(A) positive mRNA from nonmalignant cells, from retrovirus infected and transformed cells, and chemically transformed cells of human origin for the absence or presence of poly(C) and poly(G) sequences.

Planned DNA studies: (1) Location of the poly(dC) and poly(dG) sequences in subunit-length cDNA copies of retrovirus RNA. (2) Continued examination of the DNA from nonmalignant cells, from retrovirus infected and transformed cells, and from chemically transformed cells of human origin for the absence or presence of the poly(dC) and poly(dG) sequences. (3) Determination of the kilobase length of the cDNA transcripts when oligo(dA), oligo(dC), and oligo(dG) are used as primers in the in vitro synthesis of cDNA.

Planned glycoprotein studies: Determination of the effect(s) of known glycoprotein inhibitors on retrovirus assembly and replication.

Phase II: (1) We shall attempt to confirm the poly(I) inhibition of retrovirus replication studies of others. (2) We wish to determine whether homopolymers which are complementary to the poly(dC) and poly(dG) sequences present in the retrovirus DNA can inhibit the replication of mammalian retroviruses and to determine the precise mechanism of inhibition. (3) Also we wish to test sequence specific drugs and chemically modified antibiotics that have either a high binding affinity or intercalating ability for the poly(dC) and poly(dG) sequences for their ability, or inability, to inhibit the replication of mammalian retroviruses and to determine their precise mechanism of inhibition.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05072-02-LVC

PERIOD COVERED

September 30, 1979 to October 1, 1980

TITLE OF PROJECT (30 characters or less)

Feline Leukemia and Sarcoma Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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L. K. Donner	Visiting Scientist	LVC, NCI
J. Even	Visiting Scientist	LVC, NCI

COOPERATING UNITS (if any)

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Dr. G. Vande Woude, Head, Molecular Biology Section, LMV, NCI

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

Division of Cancer Cause and Prevention
NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The unintegrated DNA intermediates of the Snyder-Theilen strain of feline sarcoma virus (FeSV) and feline leukemia virus (FeLV, subgroup B) were analyzed by restriction enzyme analysis and were molecularly cloned in a bacteriophage lambda vector. Nucleic acid nybridization, restriction endonuclease mapping, and heteroduplex analyses of FeSV defined a sarcoma-specific DNA sequence (src) which putatively confers the properties of acute morphological transformation. Comparative studies with FeLV showed that the gene order in ST-FeSV is 5'gag-src-env-c-region-3' where only 1000 bases of gag and 600 bases of env are represented in the 4300 base FeSV genome. The gag, src, and possibly env genes encode a 78-85000 dalton polyprotein which contains the product of the FeSV src sequence. Two transformation-defective FeSV mutants were isolated which appear to synthesize altered gag-x polyproteins.

PHS-6040
(Rev. 10-76)

Objectives:

To study the structure and genetic organization of feline sarcoma viruses (FeSV); (ii) To identify the minimal genetic information required for FeSV-induced cell transformation; (iii) To elucidate the role of viral sequences in regulating the expression of recombined "src" elements acquired from normal cat cells; (iv) To study the role of FeSV encoded products implicated in the transformation process.

Methods Employed:

Extrachromosomal DNA was chromatographically purified from host cells infected with FeSV(FeLV) and used as a substrate for restriction enzyme analysis using the Southern blotting procedure. Eco RI digested viral DNA intermediates were cloned in a bacteriophage lambda vector and recombinant phages were characterized by R-looping with purified viral RNA, heteroduplexing, and restriction analyses. Portions of the FeSV and FeLV DNA intermediates were subcloned in plasmids and similarly characterized.

Viral variants isolated from cells following infection at a low multiplicity of infection were cloned under conditions favoring single virus-single cell interactions. Cells were characterized by Southern blotting of integrated proviral DNA; liquid hybridization to quantitate viral RNA expression (Crt analysis); Northern blotting procedures; radioimmunoassays for viral coded proteins; and by metabolic labeling, immunoprecipitation, and acrylamide gel electrophoresis of specifically precipitated proteins. Specific antisera were prepared to sarcoma viral gene products by inoculation of FeSV-transformed rat cells into rats and absorption of the sera with FeLV virions.

Major Findings:

Extrachromosomal DNA purified from mink cells acutely infected with ST-FeSV was electrophoretically separated, transferred to a solid substrate, and hybridized with radiolabeled DNA transcripts complementary to different portions of the FeSV and FeLV RNA genomes. DNA species of 8.5 and 5.0 kilobase pairs (kbp) in length were shown to represent the linear, unintegrated intermediates of ST-FeLV and FeSV, respectively, by the following criteria: (i) Transfection of DNA fractions containing 8.5 or 5.0 kbp viral DNA showed that the 5.0 kbp form induced foci in mink or mouse cells whereas the 8.5 kbp species gave rise to nontransforming, replicating virus in mink or cat cells. (ii) Transcripts complementary to ST-FeSV src sequences hybridized only to the 5.0 kbp species while cDNA representing sequences unique to the leukemia virus annealed selectively to 8.5 kbp DNA. (iii) The restriction endonuclease Eco RI which recognizes sites at both termini of 5.0 kbp DNA and generates an internal 4.3 kbp fragment was shown to excise a proviral DNA fragment of corresponding length from the DNA of cells nonproductively transformed by ST-FeSV. Restriction endonuclease

sites for seven enzymes were mapped within FeSV DNA and three other enzymes were identified which fail to cleave the provirus. Using DNA transcripts complementary to different portions of the FeSV genome, FeSV src sequences were positioned within 2.0-3.4 kbp from the 5' end of proviral DNA (with respect to the 5'-3' orientation of the RNA genome) and were found to be flanked by FeLV-derived sequences. Like other type C proviruses, FeSV and FeLV DNA intermediates are longer than viral RNA and contain sequences at each terminus derived from both the extreme 3' and 5' ends of viral RNA. The order of sequences can be designated 3'5'———3'5' where 3'5' signify the 0.7 kbp extended, terminally redundant (ETR) sequences and "———" represents viral structural genes.

FeSV and FeLV DNA intermediates were cloned in a prokaryotic vector and propagated in a bacterial host. DNA fractions containing 8.5 and 5.0 kbp viral DNA were cleaved in the ETR regions with Eco RI and ligated to lambda "arms" prepared from the EK-2 certified vector, λ gtWES λ B. The ligated DNA was packaged in vitro into plaque-forming phage particles, and recombinant phages containing FeSV and FeLV DNA "inserts" were isolated by "plaque-blot" techniques. The hybrid phages (designated λ ST-FeSV and λ ST-FeLV) were characterized by restriction enzyme analyses, R-looping with purified, polyadenylated viral RNA, and by heteroduplex formation. As expected, the recombinant phages contain all of the genetic information represented in FeSV and FeLV RNA genomes, are 0.7 kbp shorter than their respective proviral DNA intermediates, and contain one permuted ETR sequence. In agreement with nucleic acid hybridization studies performed with FeSV restriction fragments, src sequences were localized within 1.9-3.4 kbp from the 5' end of the DNA provirus. The FeLV-derived sequences at the 5' end of FeSV were found to originate from sequences present at the extreme 5' end of FeLV; by contrast, FeLV-derived sequences distal to the 3' end of the src gene are derived from two short, discontiguous segments of the FeLV genome.

The FeLV helper virus is known to contain 3 genes (designated gag, pol, and env in the canonical order 5'-3') plus a putatively regulatory sequence at the 3' end (the c-region). The gag, pol, and env genes code for a precursor of the internal structural proteins, reverse transcriptase, and the virion envelope glycoproteins, respectively. Heteroduplex analyses showed that the order of genes in ST-FeSV is 5'-gag-src-env-c-region-3' where only 1100 bases of gag and 600 bases of env are represented. The ST-FeSV "env" sequences appear to represent the 5' end of the ST-FeLV env gene but may encompass the pol-env junction. Comparative heteroduplexing analyses with the DNA of Moloney murine sarcoma virus (strain ml) surprisingly showed that FeSV env sequences were homologous to 300 bases of 5' env-derived sequences represented in ml MSV. Unlike ST-FeSV, the order of genes in the latter virus is 5'-gag-env-src-c-region-3'. The different topological location of 5' env-derived sequences in these two distinct mammalian sarcoma viruses appears to determine the nature of the product encoded by src sequences and may correspond to conserved regions of helper virus genomes that are required for retrovirus transforming functions.

The ST-FeSV genome was shown to code for a high molecular weight polyprotein containing antigens related to the FeLV gag gene product as well as unique antigenic determinants (designated "x") unrelated to those of FeLV. The order of polypeptides in the FeLV gag gene precursor is NH -p15-p12-p30-p10-COOH; by contrast, only p15, p12, and some p30 antigenic determinants are represented in the 78,000 dalton "gag-x" polyprotein of ST-FeSV. This implies that the x-moiety (50,000 daltons) would be encoded by 1400-1500 bases, a region corresponding almost exclusively to src sequences. These measurements are not sufficiently precise, however, to exclude the possibility that a limited number of amino acids at the carboxy-terminus of ST-FeSV gag-x are encoded by the env-derived region of the genome.

Polyproteins of the ST, Gardner-Arnstein (GA) and McDonough (M) FeSV strains were comparatively studied. Rat antisera have been prepared to the gag-x polyproteins of the three FeSV strains. Absorption of these antisera with FeLV removes antibodies to gag determinants and renders the sera specific for the x-moieties of the different FeSV polyproteins. Precipitation of radiolabeled proteins from lysates of FeSV-transformed mink or rat cells showed that ST-, GA- and M-FeSVs encode polyproteins of 78,000, 110,000, and 180,000 daltons, respectively. The x-portions of each polyprotein were found to be antigenically distinct, suggesting that the src genes of the three independent FeSV isolates are not identical. Since extensive homology has been detected between the src sequences of ST- and GA-FeSV, these results could reflect a polymorphism of src-encoded proteins similar to that observed with different strains of avian sarcoma virus, or could indicate that the complexities of the ST and GA src genes are different. Unabsorbed antisera to the ST-FeLV polyprotein did not precipitate the env gene product (gp70) from cells infected with FeLV (subgroups A, B, and C); nor did reference antisera to FeLV gp70 react with the ST-FeSV gag-x protein. The latter findings show that group-specific antigenic determinants encoded by FeLV env-derived sequences are not present in the ST-FeSV gag-x product but do not preclude the presence of type-specific envelope glycoprotein determinants.

Cat sera with reactivity to the FeLV-induced FOCMA antigen have been reported to precipitate the 85-92,000 dalton polyprotein encoded by GA-FeSV. To test whether rat antisera to the three different polyproteins would reciprocally react with polypeptides in FOCMA-positive cat cells, FL74 cells, a prototype FOCMA-positive line, were labeled with S-methionine and the lysates reacted with antisera. Analyses of the immune precipitates on SDS-polyacrylamide gels failed to demonstrate specific precipitation of products other than FeLV gag-gene precursors. Absorbtion of the sera with FeLV eliminated the reactivity to FeLV-coded products and again failed to demonstrate specific precipitation of other polypeptides. Negative results were also obtained after lactoperoxidase-catalyzed, surface-labeling of FL74 cells with ^{125}I. Thus, the data fail to support the hypothesis that the FOCMA determinant is present in gag-x. The discrepancies between

results obtained with cat anti-FOCMA and rat anti-gag-x sera can be explained by two alternative possibilities: (i) Rat antisera to FeSV gag-x polyproteins may recognize only some antigenic determinants, not including FOCMA; (ii) Of sera scored as anti-FOCMA positive by immunofluorescence of FL74 target cells, only some might contain antibodies capable of precipitating metabolically-labelled products.

In an attempt to more rigorously define sequences in ST-FeSV necessary for transformation, we took advantage of observations showing that functionally defective genomes can be identified in uncloned stocks of replication-competent viruses, and reasoned that stocks of transforming viruses might be similar. Mink cell cultures infected at low multiplicity with ST-FeSV(FeLV) were therefore cloned from single cells under conditions favoring single virus-single cell interactions. The primary colonies included (i) typical FeSV transformed nonproducer clones, one of which segregated phenotypic revertants, and (ii) FeSV-infected but phenotypically normal clones, three of which spontaneously converted at low frequency to the transformed phenotype. Revertant and focus-derived subclones were compared to sister and parental subclones expressing the opposite phenotype. Transformed subclones formed colonies in agar, produced tumors in nude mice, and failed to bind mouse epidermal growth factor, while flat sister subclones were indistinguishable from uninfected mink cells in each of these assays. Sister subclones derived from the same infectious event contained FeSV proviruses integrated at the same molecular site, regardless of which phenotype was expressed. However, one revertant subclone was identified which had lost most of the FeSV provirus but retained ETR sequences persisting at the original site of proviral insertion. This suggests that the mechanism of proviral excision involved crossover and recombination between the 0.7 kbp ETR sequences at each end of the FeSV provirus. Two flat subclones expressed viral RNA and the ST-FeSV gag-x protein. Both of these clones were susceptible to retransformation by ST-FeSV. Although unable to induce foci, the viruses rescued from these cells contained as much FeSV RNA as focus-forming viruses rescued from their transformed sister subclones. The transformation-defective (td) genomes could be transmitted to mink cells, again inducing FeSV gene products without signs of morphological transformation. These genomes represent the first described td mutants of FeSV.

Significance to Biomedical Research and the Program of the Institute:

Restriction mapping of FeSV and FeLV proviruses and molecular cloning techniques now permit detailed structural and genetic analyses of RNA tumor viruses at the level of DNA. A comparison of the different FeSV isolates, procedures for site-specific mutagenesis, and studies with transforming subgenomic fragments offer rapid and efficient means to define viral transforming genes. Concommitant studies with antisera which specifically react with the products of src genes enable the expression of these proteins to be quantitated and affords the potential of elucidating their cellular

topology and function. Data obtained in the viral system provide the necessary background for the molecular cloning of cellular *sarc* elements and their *in vitro* recombination with viral regulatory sequences. Such studies will offer the first formal proof that *onc* genes in normal cat cells have the cryptic potential to induce transformation and should provide some insight into how such genes might be activated *in situ* in normal cells.

Proposed Course:

Studies of the gag-x polyproteins of the three FeSV strains have suggested that each of these viruses is an independent isolate derived by a different recombination event involving FeLV sequences and cat *sarc* genes. Experiments are in progress to molecularly clone the GA- and MS-FeSV proviruses so that their structures can be compared to each other and to the provirus of ST-FeSV. Cellular DNA has been purified from cells nonproductively transformed with GA- and MS-FeSV, and has been digested with Eco RI. Unlike ST-FeSV, the integrated proviruses of GA- and MS-FeSV lack recognition sites for this enzyme. Thus, the GA- and MS-FeSV transformed clones, each infected with a single copy of provirus, yield unique viral DNA-containing fragments with flanking host cellular sequences. In the cells chosen for analysis, the size of these fragments is 13-15 kbp. Eco RI-restricted cellular DNA has been subjected to sequential purification by RPC-5 chromatography and preparative agarose gel electrophoresis, and has been assayed by Southern blotting for the presence of proviral DNA sequences. These methods yield a 100- to 1000-fold enrichment of viral DNA-containing fragments increasing the probability that rare recombinant phages containing them can ultimately be identified. Using methods previously employed to clone ST-FeSV DNA, the GA- and MS-FeSV proviruses will be ligated in λgtWES λB, packaged *in vitro*, and assayed by plaque-blotting techniques using nick-translated, cloned ST-FeLV DNA "probes". The resulting hybrids (λGA-FeSV and λMS-FeSV) will then be compared with cloned ST-FeSV and FeLV DNA by heteroduplex formation and by restriction enzyme analyses.

Plasmid systems have also been employed to clone subgenomic fragments of ST-FeSV and FeLV DNA. Using pBR322, subgenomic fragments representing different portions of the *gag*, *pol*, *env*, *c-region*, and *src* genes have been inserted into Bam HI, Bam HI- Eco RI, and Pst I sites in the antibiotic resistance genes of the plasmid. The recombinant plasmids were recovered by transfection of *E. coli*, identified by replica-plating in antibiotic-containing media, and verified to be FeLV or FeSV recombinants by colony hybridization techniques. Following purification of recombinant plasmid DNA, the "inserts" have been characterized by restriction enzyme analysis and back hybridization to nick-translated subgenomic fragments obtained by restriction of ST-FeSV and ST-FeLV DNA. The cloned subgenomic fragments have been nick-translated and used as probes in resolving FeSV and FeLV genes in complex DNA mixtures. In particular, a plasmid containing a 0.6 kbp *src*-specific sequence will be used as a probe in cloning the ST-related

sarc gene from cat DNA. Plasmids containing defined FeSV and FeLV restriction fragments will be supplied to other investigators interested in mapping endogenous viral elements in normal cat cells and in cat x mouse somatic cell hybrids. Transfection with cloned DNA proviruses and with recloned subgenomic fragments will be used to identify FeSV gene sequences required for transformation.

Of particular interest is the possibility of engineering viral genetic variants by site-specific mutagenesis of cloned DNA. Such genomes can be created by base substitution at sites of DNA restriction followed by recloning, and detection of altered progeny genomes by DNA transfection. Since transformation-defective viral DNAs generated by these procedures would not be expected to form foci, we propose to preinfect recipient cells with a heterologous helper virus and to assay for FeSV gene sequences in virions released from the cells. An alternative and possibly more efficacious approach would be to take advantage of recent observations that cotransfecting markers enter susceptible cells en bloc. For example, thymidine kinase (TK)-deficient 3T3 cells could be used as a cell substrate in cotransfection experiments performed with cloned herpes viral TK and viral DNA. The rare TK+ cells would be selected in HAT medium and screened directly for expression of viral gene functions. It should therefore be posrsible to define critical regions of the viral genome necessary for transformation and to obtain mutant viruses defective in various functions.

The isolation by conventional virological procedures of transmissible td ST-FeSV mutants which synthesize constituitively high levels of gag-x polyproteins suggests that these products are functionally defective. No differences in the size or degree of phosphorylation of the gag-x products have been detected in cells infected by td ST-FeSVs. Radiolabeled gag-x proteins encoded by wild-type and mutant FeSVs will be immunologically precipitated, resolved on polyacrylamide gels, eluted, and subjected to two dimensional tryptic analysis in an attempt to identify putative differences between these proteins. In addition, lactoperoxidase surface-labeling and immunofluorescence techniques are being used to assay for gag-x polyproteins on the plasma membranes of cells, in order to determine whether the polyproteins encoded by tdST-FeSV are transported to the cell surface. The latter approaches are consistent with our findings that cells infected with td FeSV, while blocked at a post-translational stage in expressing the transformed phenotype, continue to express cell surface receptors for epidermal growth factor.

Further attempts will be made to determine whether FOCMA is represented in FeSV-coded gag-x products. A battery of different antisera containing antibodies to FOCMA is being supplied by Dr. Max Essex (Harvard School of Public Health), and will be scored "blind" for immunofluorescent reactivity with both cat FL74 and FeSV-transformed mink cells. If immunofluorescent titers are shown to correlate in the two systems, the ability of these sera to precipitate gag-x products will be reevaluated by metabolic labeling and

immunoprecipitation procedures. Suitable sera will then be tested for reactivity with control transformed cells, td FeSV-infected but phenotypically normal cells, and with clones obtained by transfection with FeSV subgenomic fragments or engineered viral variants. Such studies should help to elucidate the nature of this antigen and whether it is indeed encoded by FeSV src or type-specific FeSV/FeLV gag or env sequences.

Publications:

Sherr, C. J., Fedele, L. A, Donner, L., and Turek, L. P.: Restriction endonuclease mapping of unintegrated proviral DNA of Snyder-Theilen feline sarcoma virus: localization of sarcoma-specific (src) sequences. J. Virol., 32: 860, 1979.

Sherr, C. J., Fedele, L. A., Oskarsson, M., Sullivan, M., and Vande Woude, G. F.: Comparative structures of unintegrated DNA intermediates of Snyder-Theilen feline leukemia and sarcoma viruses cloned in bacteriophage λ. In Viruses in Naturally Occurring Cancers. New York, Cold Spring Harbor Press, in Press.

Sherr, C. J., Fedele, L. A., Oskarsson, M., Maizel, J., and Vande Woude, G. F.: Molecular cloning of Snyder-Theilen feline leukemia and sarcoma viruses: Comparative studies of feline sarcoma virus with its natural helper and with Moloney murine sarcoma virus. J. Virol., 34: 200-212.

Ruscetti, S. K., Turek, L. P., and Sherr, C. J.: Three independent isolates of feline sarcoma virus code for three distinct gag-x polyproteins. J. Virol., in Press.

Donner, L., Turek, L. P., Ruscetti, S. K., Fedele, L. A., and Sherr, C. J.: Transformation-defective mutants of feline sarcoma virus which express a product of the viral src gene. J. Virol., in Press.

Ruscetti, S. K., Donner, L., and Sherr, C. J.: A model for FOCMA expression in Cells transformed by feline leukemia and sarcoma viruses. In Fields, B., Janenisch, R. and Fox, C.F. (Eds.): Animal Virus Genetics, ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. 18. New York, Academic Press, in Press.

Sherr, C. J., Donner, L., Fedele, L. A., Turek, L., Even, J., and Ruscetti, S. K.: Molecular structure and products of feline sarcoma and leukemia viruses: Relationship to FOCMA expression. In Hardy, W.D., Essex, M. and McClelland, A.J. (Eds.): Feline Leukemia Viruses. Proceedings of the Third International Symposium on Feline Leukemia. New York, Elsevier North-Holland Inc., in Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05078-02-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Animal Models for EBV Infection in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	P.H. Levine	Medical Director	LVC NCI
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LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Clinical Studies Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Immunologic techniques were applied to the study of EBV oncogenesis in marmosets. Tumors were produced in a white-lipped and common marmoset. A specific cellular immune response to EBV, the appearance of serum blocking factor, and elevated EBV antibodies were associated with the appearance of tumor in the white-lipped marmoset. Four rabbits inoculated with Herpesvirus saimiri developed malignant lymphoma within two months of inoculation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To develop an animal model for EBV infections in man.

Methods Employed:

B95-8 Epstein-Barr virus (EBV) was inoculated into the bone marrow (BM) and peritoneum (IP) of six white-lipped marmosets (WLM). Heparinized blood was collected at 14 day intervals for serological and cellular immunity assays. Common marmosets were inoculated into BM and IP with EBV and TPA. Inbred strain III/J rabbits were inoculated with Herpesvirus saimiri (HVS) intravenously.

Major Findings:

One of six WLM developed diffuse malignant lymphoma 55 days post-inoculation. Autopsy revealed enlarged maxillary, mandibular, cervical and mesenteric lymph nodes. Histopathology of these lymph nodes showed well differentiated lymphocytes with an overlying area of inflammation and necrosis. The normal architecture of the lymph nodes was replaced by the infiltrating lymphocytes which included numerous cells with mitotic figures and degernating nuclei. EBV DNA was detected in one of the enlarged lymph nodes. The affected animal had high EBV VCA and EA antibodies. The animal developing diffuse malignant lymphoma had a significant CMI response to EBV antigens and to PHA, which became depressed as the tumor progressed. Serum obtained from the affected animal during this period had a blocking factor which decreased or blocked the response of monolayer cells from clinically normal animals to PHA. One common marmoset inoculated with EBV and TPA developed a fatal lymphoproliferative disorder. EBV DNA was detected in the affected animal's lymph nodes. Four of four rabbits inoculated with HVS developed malignant lymphomas.

Significance to Biomedical Research and the Program of the Institute:

EBV has been implicated as causing a variety of diseases in humans, ranging from inapparent infection to lymphoma. The development of an animal model for EBV infection is important to studies on the etiology, host-virus interactions and control of EBV associated diseases. Although cotton-topped marmosets (CTM) are the most susceptible for EBV induced lymphomas, an alternate animal model is necessary because CTM are an endangered species available in limited numbers for biomedical research. WLM are available in considerable numbers. This study not only suggests WLM as an alternate animal model for EBV but also demonstrates the feasibility of investigating cell mediated immune response in these animals. This animal model will be useful in further investigating the pathogenesis of EBV, the effect of co-carcinogens, and the effectiveness of antiviral agents in the control of EBV-induced malignancy. The demonstration that rabbits are susceptible to oncogenic herpes viruses provides additional cost-effective animal models for EBV-induced lymphoma.

Proposed Course:

We intend to determine the relative importance of age and route of inoculation in the susceptibility of WLM to EBV oncogenesis. We will also study whether BM inoculation of other nonhuman primates will accelerate the oncogenic process. In addition, we will study the susceptibility of III/J inbred rabbits to EBV oncogenesis.

Publications:

Sundar, K.S., Ablashi, D.V., Bengali, Z.H., Levine, P.H. and Nonoyama, M.: Mitogenic effect of 12-O-tetradecanoyl-phorbol-13-acetate on non-human primate mononuclear cells and in vitro interaction with EBV. Arch. of Virol. (in press).

Ablashi, D.V., Bengali, Z.H., Eichelberger, M.A., Sundar, K.S. and Levine, P.H.: Increased infertility of primate oncogenic herpesviruses with tumor promoter12-O-tetradecanoyl-phorbol-13-acetate. Proc. Soc. Exp. Biol. Med. (in press).

Ablashi, D.V., Sundar, S.K., Armstrong, G.R., Galoway, P., Valerio, M., Bengali, Z.H., Kemp, J., Fox, R.R.: Herpesvirus saimiri induced malignant lymphoma in inbred strain III/J rabbits (Oryctolagus cuniculus). J. Cancer Res. & Clin. Oncol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05079-02-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Virus Protein Interactions and Organization During Viral Development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Anthony Demsey Expert LVC NCI

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SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is the determination of the viral proteins, their organization and interactions required for MuLV development. Employing a temperature-sensitive mutant of RMuLV, transmembrane associations of viral envelope and core components were demonstrated prior to budding, although budding appeared at times to occur without viral gp70. This latter phenomenon was confirmed in a cell line (HTG2) of hamster origin which is totally lacking gp70. In addition, the core polyprotein of HTG2 virus, which usually remains uncleaved, was shown to undergo cleavage during incubation with disrupted avian myoblastosis virus. The presence of gp70 in an aberrant form also permits virus budding as seen in B16 melanoma cell lines in which gp70 antigenicity is present in an 80,000-85,000 MW protein(s). Finally, studies were begun to determine the effects of interferon on virus production and glycoprotein expression in Swiss/3T3 cells infected with MoMuLV. Additional studies on VSV-infected L cells after interferon treatment showed progressive loss of glycoprotein, and therefore virus spikes, with increasing (5-30 units/ml) interferon concentrations.

Project Description

Objectives:

To define which interactions of virus components at the cell surface are required for virus production and subsequent infectivity, and to determine how altering these interactions affects virus organization.

Methods Employed:

Tissue culture, electron microscopy, fluorescence microscopy, gel electrophoresis, radioimmunoprecipitation, autoradiography, radioisotopic assays, antibody-mediated labelings, column chromatography, virus isolation and component purification.

Major Findings:

The major glycoprotein component of the murine leukemia virus (muLV) envelope, gp70, mediates infectivity, accounts for virus hemagglutinating activity, possesses type, species, and interspecies antigenicities, and has the capacity to induce production of neutralizing antibody and to confer effective immunity to MuLV-induced leukemias. These molecules are also known to aggregate into 10 nm-diameter globular knobs which are concentrated on the virus surface, and are present, as well, dispersed on the generalized surface of MuLV-producing cells. However, the manner in which these molecules arrive at the cell surface, associate with each other and with other virus (and host) proteins, and are organized into knobs is not certain. Furthermore, the precise importance of this organization in respect to infectivity, to antigen-antibody interaction, and ultimately to tumor survival and escape mechanisms is yet to be investigated.

We have demonstrated that cells infected with ts25 virus, a temperature-sensitive mutant of Rauscher MuLV (RMuLV), provide an ideal system for studying certain molecular interactions at the cell surface, since molecular assemblies containing MuLV-specified envelope components can be viewed directly on infected cell surfaces without the need for introducing a potentially perturbing identifying ligand. Certain changes at the cell surface due to ts25 infection and maintenance at 39^{0} are detectable: (a) in carbon/platinum surface replicas, 10 nm knobs containing viral glycoprotein appear on the cell surface, and sometimes aggregate into patches which often protrude slightly from the cell surface; (b) in thin sections, submembranous densities are found, often underlying parts of the cell surface which also protrude. We found that labeling the cell surface, with antiserum directed against the virus surface glycoprotein and with ferritin, resulted in localization of label above the submembranous densities. Furthermore, employing labeling of sectioned cells, with antiserum directed against the major viral core protein and with ferritin, we identified these submembranous densities as

viral core material, suggesting a transmembrane association between virus envelope and core components. In other experiments, upon transfer of cells from 39° to 31°, virus buds appeared within 30 sec, and release continued even if cells were returned to 39°. While bud formation can occur at 0° without virus release for up to 4 hours at that temperature, upon transfer to 39° a burst of virus release occurs, again indicating that release is an energy-dependent step. Often, buds form within knob patches, but are not restricted to these areas. Furthermore, incorporation of knobs into buds does not appear to be a requirement for bud formation.

A similar finding was the lack of knobs on all virus produced by HTG2 hamster cells. Subsequently, it was determined by immunoelectron-microscopy and by radioimmunoprecipitation that virus envelope proteins gp70 and p15E are totally absent from the virus and cell although the distribution of IMP is not different from cells infected with wild-type virus. Thus the lack of knobs appears to have little effect on the disposition of other intrinsic membrane proteins, even within virus buds themselves. Possibly as a result, the virus envelope appears to fit very loosely around the core. The organization of the HTG2 core itself is also interesting in that it retains the "immature" structure, and the core polyprotein precursor fails to undergo cleavage. Thus, the only viral protein, p65, was shown to possess p30, p15, and p12 antigenicities although p10 was lacking and apparently substituted with another 10,000 MW peptide. We have recently determined that incubation of HTG2 virus with RMuLV or Moloney MuLV (MoMuLV), in the presence of 0.5% NP40 to disrupt the virions, will not cause cleavage of p65, but that incubation with avian myoblastosis virus for 1 hr at 37° will yield a number of cleavage products, principally: p42 which includes p30 and p12, possibly a p40 containing p30 and "p10", p26-28 which includes p15 and p12, and p15 alone. Although the avian RNA tumor virus protease is reported to differ significantly from the MuLV protease, it is nevertheless surprisingly active in this system.

In contrast to the lack of gp70 on HTG2 virus, in certain instances gp70 antigenicity appears to be present on some MuLV in somewhat different molecular weight forms. One such case under study is the B16 melanoma cell system. We have determined that the parent B16 cell line, as well as its metastatic variants, produce C-type virus having an apparently normal complement of knobs, but that the gp70 antigenicity appears, primarily or exclusively, in a stable 80,000-85,000 MW protein(s). While gp80-85 reacts with anti-gp70 serum, an anti-p15E serum has not proven similarly reactive, but this may reflect only a type specificity not yet determined. Although the presence of this gp80-85 appears uniform for all B16 variants, certain other, lower molecular weight proteins, precipitated by anti-gp70 serum, vary among the variants, and their effects on differences in metastatic potential of the variants must be considered.

Finally, recent studies have investigated the effects of interferon on murine mammary tumor virus production in Mm5mt/c$_1$ cells, and on MoMuLV production in Swiss/3T3 cells. In both cases, 100-300 U/ml concentrations of interferon resulted in an increase of virus on the cell surface. However, it appeared that the vast majority of virus particles were already budded from the membrane, having, in the case of MoMuLV, lost a significant number of knobs as well as their "immature" core structure.

Thus, most viruses seemed to have lost membrane attachment, but remained adherent in some fashion to the cell surface. Furthermore, the interferon-treated cells often appeared to be more villous and rounded than controls, and it was on such cells that enormous numbers of virus were found. The possibility that virus production might vary with stages of the cell cycle was considered, and led to (1) the study of virus production in synchronized cells, and to (2) the finding that interferon does appear to have an effect on cell cycle in these systems. In addition, we have recently determined that a defect in virus surface glycoprotein organization is responsible for the decrease in infectivity of Vesicular Stomatitis virus (VSV) produced in L cells after treatment with low concentrations of interferon (5-30 reference units/ml). This is supported by electron microscopic evidence indicating that the glycoprotein-containing virus spikes are progressively depleted on VSV produced under these conditions. This information is furthermore interesting because higher interferon concentrations reportedly act upon the VSV-infected cell to inhibit virus-directed translation or transcription. It is possible, therefore, that subtle, and multiple, effects are the result of interferon treatment in some systems.

Significance to Biomedical Research and the Program of the Institute:

Important to our understanding of viral effects on the host - whether the result is productive infection, latency, or transformation - is our understanding of the mechanisms of transmittance, infection, and virus development. In regard to the retroviruses, central to this aim is our knowledge of the precise interplay between host and virus components at the membrane which enables virus budding to occur, as well as of transmembrane mechanisms. Through a multifaceted examination of virus surface antigens, including our unique approach to visual cell surface analysis, we hope to obtain significant information regarding (a) the nature and properties of cell-surface and viral-envelope membranes, (b) the dynamic behavior of proteins within these membranes and with viral core proteins, and (c) the various mechanisms involved in permitting or retarding virus development and infectivity. In addition, the ability to detect such cell-surface changes may be translated to a number of related fields and would be particularly helpful in understanding changes which occur when normal cells become transformed. This approach has already provided significant information regarding MuLV assembly, native organization of MuLV gp70 in the cell surface membrane, transmembrane relationships between gp70 and virus core components, and interactions between individual gp70-containing assemblies during antibody-induced "modulation" that could not be obtained in the same way by

any other procedure. Such combined procedures should also yield interesting information on the details of MuLV antigen-antibody interactions at the cell surface. Furthermore, we have devised a procedure for analysis of cell surface distributions of gp70-containing assemblies to such a point that deviations from random distributions can be quite precisely defined and compared. In addition, Interferon effects on B 16 cell surface virus production and/or glycoprotein may provide an interesting model for probing the bases for metastatic properties. Eventually such an approach should give unprecedented information concerning the role of antibody interactions at the cell surface in tumor survival, metastasis, and control.

Proposed Course:

Continuing studies with ts25-infected cells will center on the involvement of cytoskeletal elements in virus budding. With HTG2 virus, the specific factors contributing to core precursor cleavage by AMV will be investigated, along with concomitant morphological alterations. Further studies on the B 16 melanoma cell lines will be concerned with the importance of viral antigens, aberrations, and interferon effects on tumor survival and metastasis. The specific effects of interferon on MuLV-infected and VSV-infected cells will also be pursued.

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Gershon, N.D., Demsey, A. and Stackpole, C.W.: Analysis of local order in the spatial distribution of cell surface molecular assemblies. Exptl. Cell Res. 122: 115-126 (1979).

Demsey, A.: Structure and composition of a noninfectious C-type retrovirus produced by a cell line established from a MSV-induced transplantable hamster tumor. In Baily, G. W. (Ed.): Proc. Microsc. Soc. of Amer. Baton Rouge, La., Claitors Publishing Div., 1979, p. 42-43.

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Grimley, P.M. and Demsey, A.: Dynamics of cell membranes during virus infection. In Trump, B. F. and Arstila, A. U., (Eds.): Pathobiology of Cell Membranes, Vol. II. New York, Academic Press, (in press.)

Demsey, A., Kawka, D., Galuska, S., Margulies, I. and Heine, U.: Early events of C-type virus budding in cells infected with a Rauscher leukemia virus temperature-sensitive mutant. Arch. of Virol. (in press).

Demsey, A.: Early events in virus budding: murine leukemia virus envelope glycoprotein - core protein organization at the cell surface. In Bailey, G. W. (Ed.): Proc. Microsc. Soc. of Amer. Baton Route, La., Claitors Publishing Div. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05080-02-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cell Biology on Viral Oncogenesis: Mechanism of Transformation and Tumorigenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Hatanaka	Expert	LVC NCI
Other:	K. Tsukamoto	Visiting Scientist	LVC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the project is to understand the mechanism of in vitro transformation and in vivo tumorigenesis by tumor viruses.

A. We have succeeded in isolating the transformed and tumorigenic Indian muntjac cells infected by a tumor virus with stable diploidy (2n=6 in the female and 7 in the male).

B. A muntjac gene segment was inserted into hamster chromosomes by cell fusion.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

A. To investigate the properties of Indian muntjac cells transformed by a sarcoma gene.

B. To transfer some gene segments by cell fusion from one species to another one.

Methods Employed:

A. Cell cultures in plastic plates and colony formation in soft agarose culture. Standard biochemical and cell biological technique to determine biological markers in transformants. Transplantation of the transformed cells in nude mice.

B. Nucleic acid hybridization assays, including hydroxyapatite chromatography, Southern transfer and hybridization to blots. Restriction enzyme treatment to cleave cell DNA and gel electrophoresis.

Major Findings:

It is commonly observed that animal cells become first tetraploid and then aneuploid or hyperploid after multiple passages in culture. Most established mouse cell lines, for instance, have a near tetraploid karyotype, even if the cells maintain a nontransformed morphology. It would be more difficult to find transformed cells with stable diploidy.

Indian muntjac (Muntiacus muntjak) cells have a diploid chromosome number of 6 in the female and 7 in the male, the lowest numbers yet described in a mammal and maintain diploidy after many passages in culture. We have successfully transformed these cells by a xenotropic murine sarcoma retrovirus, 43-2XV and isolated the morphologically transformed clones of male and female Indian muntjac that retained a stable diploid chromosome number.

It is advantagenous to employ these diploid clones of Indian muntjac to study expression of the host and viral genes compared to using polyploid or aneuploid transformants, because consideration of active, silent and missing chromosomes, or gene dosage effects can be minimal. Also, the Indian muntjac chromosomes have several physical and numerical advantages which aid in identifying and separating the assigned chromosome(s) for study of viral gene integration and expression. The study of these genetically advantageous cell lines has been a major researach project of this section.

A. The transformed clones of Indian muntjac cells derived here have several unique characteristics that are not easily found in the other animal systems. First, all the transformed clones maintain a stable diploid karyotype. This observation is quite rare in experimental animal systems as far as we are

aware. In addition to the transformed morphology, the clones revealed rapid growth, with no contact inhibition of growth as observed by other cells transformed by physical, chemical and biological agents. However, no anchorage independency has been developed by the muntjac transformants through conventional soft agar methods. However, the addition of a human lymphoma cell line, Raji-TK$^-$ within the chemically conditioned media (hypothxanthine-aminopterine-thymidine (HAT), enabled to form colonies from these diploid transformants. The chemical nature of this promoting factor(s) for colony formation of diploid cells remains to be elucidated.

B. These characteristics of muntjac cells seem to be very favorable for the study of cell-cell hybrids because they will make it easy to discern muntjac chromosomes in the hybrid cells.

Irrespective of these advantages, no hybrid cells have been reported between muntjac cells and cells from other species so far, although there is one report in which muntjac cells were used for the analysis of premature condensation of chromosomes after fusion of them with human cells.

Indian muntjac diploid cells that have only three pairs of easily discernible large chromosomes were fused with hamster cells deficient in hypoxanthine guanine phosphoribosyltransferase (HGPRT) using polyethyleneglycol. Cells that survived in hypoxanthine-aminopterine-thymidine (HAT)-ouabain medium were analyzed.

Usual hybrid cells containing both of the muntjac and hamster chromosomes in a given cell were not found at all. Instead, cells had the same chromosomal sets as those of either parental muntjac or hamster cells.

A clonal isolate that had the same chromosomal sets as those of parental hamster cells was analyzed in detail and was found to show the following chracteristics: (i) the survival curve in HAT medium showed intermediate nature between those of parental cells; (ii) expressions of both muntjac and hamster antigen(s) were detected by immunofluorescence staining; (iii) the mobility of the enzyme HGPRT in the gel electrophoresis differed from that of parental hamster or muntjac cells. These results indicate that the clonal isolate (AD202h) is a somatic cell hybrid of hamster and muntjac which contains chromosomal sets of hamster with an inserted segment of muntjac gene including HGPRT.

Significance to Biomedical Research and the Program of the Institute:

For the first time in mammalian systems, the process of neoplastic transformation can be analyzed at the genetic and chromosomal level in vitro without the compilation of heteroploid transformation.

Proposed Course:

1. The transformed clonal isolates will be further characterized in conjunction with the transformation and tumorigenesis by using biochemical and biological techniques.

2. Karyotype analyses will be extended to determine any abnormalities and translocation of chromosomes in these clonal isolates of transformants.
3. The promoting factors to form colonies in the soft agarose medium for the diploid transformants, will be characterized biologically and biochemically.

4. The gene insertion shown in muntjac-hamster cell fusion can be extended to other gene engineerings.

Publications:

Hatanaka, M. and Klein, R.: Tumorigenicity of Indian muntjac diploid cells by the proviral integration of sarcoma gene of a mouse retrovirus. J. Exp. Med. 150:1195-1201, 1979.

Tsukamoto, K. and Hatanaka, M.: Insertion of muntjac gene segment into hamster cells by cell fusion. J. Cell. Phys. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05097-02-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Isolation and Characterization of Growth Factors and Binding Proteins Derived from Human Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Marquardt	Visiting Scientist	LVC	NCI
OTHER:	G. J. Todaro	Medical Officer	LVC	NCI
	J. E. De Larco	Expert	LVC	NCI
	J. Keski-Oja	Visiting Fellow	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project seeks to purify and characterize the major classes of growth factors released by transformed human cells. This includes chemical and biological comparisons with known hormones like human somatomedins and animal cell growth factors such as Epidermal Growth Factor, Sarcoma Growth Factor, etc.). A human fibrosarcoma cell line (8387) produces a growth factor related to multiplication stimulating activity (MSA) produced by a rat liver cell line grown in serum-free medium. MSA belongs to the insulin-like growth factor family of the somatomedins and shares interacts with the same receptors in various tissues. The 8387 growth factor and its specific binding protein were isolated. Comparisons were made with known human somatomedins and MSA-like growth factors from various tumor cell types. The purified binding protein was used to develop a human specific competitive binding protein assay to screen human tumor tissues for MSA-like material.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To purify and characterize the major classes of growth factors released by transformed human cells in order to chemically and biologically compare the isolated polypeptide hormones with human somatomedins and other known animal growth factors (such as EGF). This laboratory recently presented evidence that a line of human fibrosarcoma cells (8387) produces a growth factor related to multiplication stimulating activity (MSA), a growth factor which was first described as a product of a rat liver cell line grown in serum-free medium. MSA is a member of the insulin-like growth factor family, the somatomedins, and shares with these polypeptides the ability to interact with the same receptor in various tissues and to stimulate growth-related processes of certain cells in culture. The rationale of these studies was to isolate the MSA-like growth factor and its specific binding protein from the supernatants of a human fibrosarcoma cell line. The growth factor could then be used to chemically and biologically compare this polypeptide hormone with known human somatomedins and MSA-like growth factors from various tumor cell types. The purified binding protein could be used to develop a human specific competitive binding protein assay to screen human tumor tissues for MSA-like material.

Methods Employed:

Biochemical and biological laboratory techniques were developed or adapted to isolate and purify growth factors from serum-free supernatants of human tumor cells. Radioimmunoassays were developed for the isolated growth factors. Various standard techniques were used to characterize and compare the purified growth factors on the level of the primary structure. This laboratory recently presented evidence that a line of human fibrosarcoma cells (8387) produces a growth factor related to multiplication stimulating activity (MSA), a growth factor which was first described as a product of a rat liver cell line grown in serum-free medium. MSA is a member of the insulin-like growth factor family, the somatomedins, and shares with these polypeptides the ability to interact with the same receptor in various tissues and to stimulate growth-related processes of certain cells in culture. The rationale of these studies was to isolate the MSA-like growth factor and its specific binding protein from the supernatants of a human fibrosarcoma cell line. The growth factor could then be used to chemically and biologically compare this polypeptide hormone with known human somatomedins and MSA-like growth factors from various tumor cell types. The purified binding protein could be used to develop a human specific competitive binding protein assay to screen human tumor tissues for MSA-like material.

Major Findings:

Serum-free conditioned media was used as the starting material for isolation of MSA-like growth factors from a human fibrosarcoma cell line (8387).

Laboratory techniques have been developed to process large volumes by ultrafiltration at neutral pH, utilizing the natural association of the growth factor to its binding protein. BRL-MSA competing activity in the radioreceptor assay was retained and recoveries above 85% were consistently obtained. 8387-MSA was dissociated from its binding protein at acid pH and partially purified by gel permeation chromatography on Bio-Gel P-100.

The columns were eluted with 1M acetic acid to dissociate the growth-promoting activity from the binding protein. The bulk of the protein was eluted in the exclusion volume of the column and was well separated from the growth-stimulating activity. Two major peaks of BRL-MSA competing activity were usually observed. The largest activity peak was capable of binding BRL-MSA, but not capable of stimulating growth. Fractions with the highest growth stimulating activity were pooled and further purified by carboxymethylcellulose chromatography. BRL-MSA competing activity and growth-promoting activity were determined. Three peaks of BRL-MSA competing activity were observed. The activity peak eluting shortly after application of the gradient showed extreme charge and size heterogeneity as judged by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{125}I-labeled material. The second peak of activity was occasionally absent and was not further examined. The major peak of BRL-MSA competing activity coeluted with a peak of growth-promoting activity.

The biological properties of partially purified 8387-MSA were compared in the radioreceptor assay. Both BRL-MSA and 8387-MSA competed with ^{125}I-BRL-MSA for the MSA-receptor sites of NRK cells. The slopes of the competition curves were similar although BRL-MSA was, on a molar basis, approximately 5-fold more active.

The electrophoretic protein pattern on sodium dodecyl sulfate-polyacrylamide gels reveals one major protein band, with an apparent molecular weight of 14,000, and two less intensely stained protein bands, with apparent molecular weights of 19,000 and 11,500. The 14,000 molecular weight peptide represented about 65% of the total protein input. 8387-MSA was localized by slicing gels, eluting the slices and assaying the eluates in the radioreceptor assay. BRL-MSA-competing activity coincided with the major protein band with an apparent molecule weight of 14,000.

Purified ^{125}I-8387-MSA from sodium dodecyl sulfate-polyacrylamide gels was precipitated by a rabbit antiserum against partially purified 8387-MSA. The immunoreactive 8387-MSA in carboxymethylcellulose purified preparations was localized in eluates from polyacrylamide gels in the competitive radioimmunoassay. The competing activity comigrated with the 14,000 molecular weight

protein band. Rat-MSA did not cross-react in the 8387-MSA radioimmunoassay. A competitive binding assay using 8387-MSA binding protein linked to Sepharose-4B was developed. The source of the binding protein was serum-free supernatant partially purified by gel-permeation chromatography on BioCel P-100 in 1M acetic acid. The displacement of binding of ^{125}I-8387-MSA and ^{125}I-Rat-MSA to its partially purified binding protein by the homologous or heterologous competitor was studied. The data clearly showed that both growth factors were recognized by the same binding protein.

Significance to Biomedical Research and the Program of the Institute:

The isolation and purification of 8387-MSA has now made possible the elucidation of the primary structure in order to compare its structural features with the somatomedins and to prove or disprove whether tumor produced growth factors are different from normal peptide hormones. The development of radioimmunoassays will allow us to screen tumor tissues for MSA-like material.

Proposed Course:

The continuation of this work includes the production and purification of these growth factors in preparation of sequencing. Production of sufficient quantities will permit the study of the specific mechanism of action as well as its genetic relationship to the control of transformation, both genetically and phenotypically. Plans include the use of recombinant DNA techniques to prepare test quantities of these substances.

Publications:

Keski-Oja, J., Marquardt, H., De Larco, J. E., and Todaro, G. J.: Fibronectin releasing polypeptides from human fibrosarcoma culture media. J. Supramolec. Struct., Supplement 3, p. 195, 1979.

Keski-Oja, J., Marquardt, H., De Larco, J. E., and Todaro, G. J.: Human fibrosarcoma cells produce fibronectic-releasing peptides. J. Supramolec. Struct. Supplement 11, p. 217, 1979.

Todaro, G. J., De Larco, J. E., Marquardt, H., Bryant, M. L., Sherwin, S. A., and Sliski, A. H.: Polypeptide growth factors produced by tumor cells and virus-transformed cells: A possible growth advantage for the producer cells. Hormones and Cell Culture, Cold Spring Harbor Conferences on Cell Proliferation, Cold Spring Harbor Laboratory, Vol. 6., p. 113, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05098-02-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of the Feline Oncornavirus-associated Cell Membrane Antigen Induced by Feline Sarcoma Virus Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. H. Sliski	Staff Fellow	LVC	NCI
OTHER:	G. J. Todaro	Medical Officer	LVC	NCI
	C. J. Sherr	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)

Dr. Max Essex, Harvard University, Cambridge, Mass.

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project if to further characterize the feline oncornavirus-associated cell membrane antigen (FOCMA) expressed on feline sarcoma virus (FeSV) transformed cells. Specifically, (1) the expression of FOCMA on non-producer FeSV transformed cells of various species was defined by membrane immunofluorescence, (2) FOCMA was characterized biochemically to determine its size and whether there were determinants unique from the virus structural proteins, (3) hybridomas producing monoclonal antibody to FOCMA were developed in order to purify the protein and to employ the antibodies in an immunotherapy program for feline leukemia, and (4) to determine if FOCMA is a true tumor specific antigen and whether it is encoded by the virus and induced upon transformation independently of virus replication.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To further characterize the feline oncornavirus-associated cell membrane antigen (FOCMA) expressed on feline sarcoma virus (FeSV) transformed cells. Specifically, (1) to define by membrane immunofluorescence the expression of FOCMA on non-producer FeSV transformed cells of various species, (2) to characterize FOCMA biochemically to determine its size and whether there are determinants unique from the virus structural proteins, (3) to develop hybridomas producing monoclonal antibody to FOCMA in order to purify the protein and to employ the antibodies in an immunotherapy program for feline leukemia, and (4) to determine if FOCMA is a true tumor specific antigen and whether it is encoded by the virus and induced upon transformation independently of virus replication.

Methods Employed:

Standard methods for cell culture, virology and microbiology were used. Biochemical methods were used for the isolation and purification of antigens. The sera were tested on feline lymphoma cells in an indirect membrane immunofluorescence test. Radioimmunoassays were developed or adapted. Published techniques were adapted for the development of hybridomas for the production of monoclonal antibodies.

Major Findings:

A. Expression of FOCMA by Membrane Immunofluorescence on FeSV Transformed Cells.

The isolation of nonproducer FeSV-transformed mink cells has made it possible to test for FOCMA expression in cells of a heterologous species in the absence of virus production. Three independently isolated cell lines of FeSV-transformed mink lung cells (Mvl-Lu) were used: each had been transformed with the Gardner-Arnstein strain of FeSV. Our data indicate that FOCMA expression is dependent on transformation by FeSV, and independent of the presence of viral structural proteins. In association with previous results in-vivo, FOCMA, therefore; appears to be induced by transformation events associated with the expression of either FeLV or FeSV. We believe this is the first report describing a tumor virus-associated antigen on nonproducer tumor cells that is known to be immunogenically effective under natural condition.

The data from these mink cell lines have demonstrated that FOCMA production correlates with morphologic transformation specifically induced by FeSV, and

is independent of the presence of viral structural proteins on the plasma membrane. Previous in-vivo data have indicated that FOCMA occurs on the plasma membranes of feline lymphoid cells transformed by FeLV. Together, these results demonstrate that the expression of FOCMA parallels transformation induced by either FeLV or FeSV. Therefore, FOCMA appears to be the first described nonvirion tumor-specific surface antigen that is induced by a naturally occurring oncornavirus of an outbred mammalian species.

Mink cells transformed by FeSV are positive for FOCMA, however, there have been two independent reports on the isolation of endogenous mink viruses which are related to the feline leukemia virus (FeLV). The endogenous mink viruses can be activated either by continuous passage, or co-cultivation techniques. Measured by radioimmunoassay, the mink endogenous virus p30 is antigenically related to p30 of FeLV. Both FeLV and the mink endogenous virus contain polymerases which are related to endogenous rodent virus polymerase, particularly rat. It has been theorized that an ancestral rat virus was independently transferred to the cat and the mink. Due to the relatedness of FeLV and the endogenous mink viruses there remained a possibility that FOCMA expression in FeSV transformed mink cells may be due to the mink virus or derepression of other endogenous related sequences and not actually be clear evidence that FOCMA is encoded by FeSV.

Cells from rat, tree shrew, dog, lemur and mouse were therefore examined for FOCMA expression using the indirect membrane immunofluorescence test. These species are distantly related to each other and to mink and cat. The results using cells of other species agreed completely with those of the mink and cat. All cells transformed by FeSV were positive for FOCMA, whether or not they were virus producers. The FeSV transformed rat cells, F3NRK, did not react above very low levels of non-specific activity, with the cat anti-FOCMA serum. Unlike the mink, dog, and tree shrew cells from which FeSV can be easily rescued, the rat cell FeSV is not readily rescueable. FOCMA antisera of rat and rabbit origin does react with the FeSV transformed rat cells. This may reflect a difference in externalization of FOCMA on the rat cells versus other species cells.

B. Biochemical Characterization of FOCMA.

The expression of FOCMA in FeSV-transformed rat and mink nonproducer cells indicated that this antigen is encoded by a feline type-C viral gene. Following rescue of the FeSV genome from nonproducer mink or rat cells, FOCMA and FeLV-related p15 and p12 antigens can be detected in extracellular pseudotype particles. The structural proteins p15 and p12 are packaged in equimolar amounts that correlate with the amount of FOCMA activity. Under nondenaturing and denaturing conditions, p15, p12, and FOCMA cochromatograph with phosphorylated molecules of 85,000 daltons (p85). Molecules of this size can be precipitated by both antiviral and anti-FOCMA antibodies, and FOCMA specifically binds to immunoadsorbants prepared with antibodies to

FeLV. Because FeLV proteins do not themselves react with antibodies to FOCMA, we conclude that FOCMA differs from p15 and p12 but is associated with viral antigens in molecules of 85,000 molecular weight. Our data do not formally exclude the possibility, however, that more than one 85,000 molecular weight species is encoded by FeSV.

Cat sera containing antibodies to FOCMA were adsorbed with disrupted pseudotype particles, and the adsorbed sera were tested by immunofluorescence with nonproducer, FeSV-transformed mink cells (64F3CL7). FeSV pseudotypes decreased the anti-FOCMA titer, and the degree of reduction corresponded to the quantities of FeLV p15 and p12 antigens detected by radioimmunoassay. KiSV (M7), Moloney MSV (M7), and different helper viruses did not decrease the anti-FOCMA titer. Purified viral proteins from FeLV (including p15 and p12), RD-114, or MuLV did not affect the anti-FOCMA reactivity.

Since FOCMA expression on FeSV-transformed nonproducer cells and FeLV-infected lymphoid tumor cells may not be identical, we have designated these antigens FOCMA-S and FOCMA-L, respectively. Because neither FeLV, p15 nor p12 react with cat antibodies to FOCMA, FOCMA-S appears to reside in a unique domain of p85 which lacks p15 and p12 amino acid sequences.

We have now developed a radioimmunoasay for FOCMA-S using reagents generated entirely outside of a cat cell system. FeSV pseudotype virions were used to prepare rabbit antisera directed toward FOCMA and also as a source of FeSV p85 protein. Using appropriately adsorbed antisera, this assay detects FOCMA but not the major structural proteins of FeLV. Although the polyprotein (p85) detected in pseudotype virions contains p15 and p12 antigenic determinants, antibodies to these can be adsorbed. The RIA for FOCMA-S does not detect then, the major viral structural protein antigens found in p85.

While FOCMA-S was readily scored in FeSV pseudotypes, it was marginally detected in FeSV (FeLV) preparations of equivalent focus-forming titer grown in cat cells. This is consistent with the failure to detect significant quantities of ^{32}P-labeled p85 molecules in such stocks. In cells, both p85 and its p60 derivative contain FOCMA-S determinants but only p85 is packaged in rescued pseudotype virions. Thus, the specificity for packaging the polyprotein appears to reside in the p15-p12 moiety. The reduced concentration of p85 in FeSV (FeLV) may then reflect an increased rate of processing in the presence of homologous helper virus.

The RIA detects FeSV pseudotypes but not their helper viruses nor FeLV itself. Of the known FeLV structural proteins, purified viral gp70, p30, p15 and p12 were directly tested and did not compete for FOCMA-S determinants in the p85 tracer antigen. The absence of competition using FeLV itself further suggests that FeLV p10 lacks FOCMA-S determinants since the competing FeLV preparation were of very high titer and should have contained high concentrations of this protein. However, it is difficult to exclude

the presence of FOCMA-S determinants in viral reverse transcriptase since the enzyme represents only a small fraction of the total virion protein. High titer antisera raised to the polymerase of FeLV and adsorbed with p15 and p12 proteins do not react with ^{125}I-p85; nor do rabbit antisera to p85 inhibit FeLV polymerase activity. Taken together, the results suggest that the RIA detects only a sarcoma virus-specific antigen.

Significance to Biomedical Research and the Program of the Institute:

FOCMA has been shown to be encoded by FeSV, and is transformation specific and distinct from the virus structural proteins, making it one of the most attractive candidates for a "transforming protein" in any mammalian retrovirus system. The cat is an outbred, free-living mammal. Under natural conditions FeLV-FeSV produce lymphomas, leukemias and fibrosarcomas. FOCMA is the target for tumor immunity and is expressed on virus negative tumor cells whether induced by FeLV or FeSV. FOCMA under natural conditions is highly immunogenic, and an immune response to FOCMA protects the animal against development of tumors. These studies on the biochemical purification of FOCMA may lead to a vaccine which would protect cats against feline leukemia and sarcoma. They would also allow testing of the transformation capacity of FOCMA.

Proposed Course:

This studies will be continued as outlined under the objectives described above.

Publications:

Essex, M., Sliski, A. H. and Hardy, W. D. Jr. FOCMA: A transformation specific RNA sarcoma virus encoded protein. In P. Chandra (Ed.): Mechanism of Neoplasia. New York, Plenum Press, 1979, pp. 125-138.

Essex, M., Grant, C. K., Sliski, A. H. and Hardy, W. D. Jr. Feline leukemia and immunological surveillance. In P. Chandra (Ed.): Mechanism of Neoplasia. New York, Plenum Press, 1979, pp. 427-438.

Sliski, A. H. and Essex, M. Sarcoma virus-induced transformation specific antigen: Presence of antibodies in cats that were naturally exposed to leukemia virus. Virology 95:581-586, 1979.

Essex, M., Grant, C. K., Cotter, S. M., Sliski, A. H,. and Hardy, W. D. Jr. Leukemia specific antigens: FOCMA and immune surveillance. In R. Neth, R. Hofschneider and K. Manweiler (Eds.): Modern Trends in Leukemia III, New York, Springer-Verlag, 1979, p. 272.

Essex, M., Sliski, A. H., Worley, M., Grant, C. K., Snyder, H. Jr., Hardy, W. D. Jr. and Chen, L. B.: Significance of the feline oncornavirus-associated cell membrane antigen (FOCMA) in the natural history of feline leukemia. I Cold Spring Harbor Symp. Quant. Biol. Vol. 45 (in press).

Snyder, H. W. Jr., Phillips, J. J., Hardy, W. D. Jr., Zuckerman, E. E., Essex, M., Sliski, A. H. and Rhim, J.: Isolation and characterization of proteins carrying the feline oncornavirus-associated cell membrane antigen (FOCMA). I Cold Spring Harbor Symp. Quant. Biol., Vol. 45, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1-CP-05099-02-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Evolution, Sequence Organization and Biological Activity of Endogenous Retroviruses of Old World Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The distribution within primates of nucleic acid sequences related to endogenous retroviruses, their sequence organization and the factors influencing their biological activity was studied. Specifically, (1) to detect and characterize the virus-related sequences in primates and the factors involved in their evolution; (2) to develop probes appropriate for detection of virus-related sequences in human cells, (3) to characterize the organization of the 50-100 copies of endogenous sequences through restriction mapping and recombinant DNA techniques; (4) to correlate the extent of viral expression with the state of integration of viral sequences, both endogenous and exogenous, through restriction mapping and transfection; (5) to investigate other factors involved in the expression of viral sequences in primates.

Project Description

Objectives:

To characterize the distribution within primates of nucleic acid sequences related to endogenous retroviruses, their sequence organization and the factors influencing their biological activity. Specifically, (1) to characterize the virus-related sequences in primates and the factors involved in their evolution; (2) to develop probes appropriate for the detection of virus-related sequences in human cells; (3) to characterize the organization of the 50-100 copies of endogenous sequences through restriction mapping and recombinant DNA techniques; (4) to correlate the extent of viral expression with the state of integration of viral sequences, both endogenous and exogenous, through restriction mapping and transfection, (5) to investigate other factors involved in the expression of viral sequences in primates.

Methods Employed:

The existence of endogenous viruses indicates that similar viral genomes or related sequences may exist in human cells. Since we would ultimately like to characterize such human viral sequences we have focused on the viruses isolated from man's closest relatives, the Old World monkeys. These viruses fall into three distinct classes: (1) the various closely related isolates of type-C virus from several species of baboon (we deal with the M7 and M28 isolates), (2) a second type-C class which includes MAC-1 from stumptail macaque, CPC-1 from colobus and a related virus from rhesus and (3) the type-D virus, LAD-1, isolated from langur. Irrespective of their direct relationship to a putative human virus, these viruses compose a useful system in which to study the general characteristics of endogenous viruses. The primate system parallels the avian and rodent systems but has somewhat different characteristics from the other two systems which may be related to the fact that all the present primate viruses isolates are xenotropic.

Major Findings:

A. Detection of Virus-related Sequences in Primates and Their Evolutionary Characteristics.

We have used very low stringency hydroxyapatite techniques in order to detect sequences which are distantly related to the three classes of Old World primate viruses and to quantitate their sequence divergence. Sequences related to both type-C families diverge more rapidly in Asian primates than in African primates, but the differences between Asian and African primates are greater with the baboon virus family. In both cases, we can detect related sequences in the African apes, chimpanzee and gorilla, but not in the Asian apes, orangutan or gibbon, nor in man. In contrast, the type-D sequences

show no difference between African and Asian primates but diverge so rapidly that we cannot detect related sequences in any of the apes. The evolutionary pattern with all three viral families suggests that related sequences are present in orangutan, gibbon and man, but have diverged enough to escape detection by the present method.

The existence of two isolates of the same family, MAC-1 and CPC-1, but from relatively distant species allows us to construct an evolutionary tree for this family of viral sequences. Comparison of this tree with that derived from fossil evidence and single copy cellular DNA allows us to conclude that horizontal transmission of virus from one branch of the tree to another is not the reason for the difference between the viral sequences of African and Asian primates. Instead, the results support the hypothesis that the rate of divergence accelerates once a species leaves Africa.

The shape of the melting curves of viral cDNA hybridized to cellular DNA of the species from which the virus was isolated demonstrates that the 50-100 copies of endogenous sequence are quite homogeneous. This would not be the case if they had evolved independently for tens of millions of years. By analogy with other homogeneous, multigene families, such as the ribosomal RNA and the histone genes, we expect that the homogeneity of the multiple copies is maintained by a mechanism such as unequal crossing-over. If this were the case, we would expect that the viral copies are clustered with a repeated spacer between the copies. Restriction enzyme analyses of cellular DNA support this hypothesis.

The divergence of single copy cellular DNA between the primate species provides the background information necessary for the evaluation of the evolution of virus-related sequences. Using the low stringency hydroxyapatite techniques, we have extended existing data to include the prosimians. The resulting phylogenetic tree resolves a number of controversies as to the placement of early branch points. We unexpectedly find that the primates on Madagascar have evolved substantially slower than all other primates. This result is not predicted by the theory of neutral selection, but can be explained by several hypotheses based on the 50 million year isolation of Madagascar. Since this result may be of major importance to our understanding of DNA evolution in general, we are planning experiments to determine if carnivores also evolve slower on Madagascar than elsewhere. A positive result would eliminate the rather trivial explanation that the slow down results from a founder effect in which the original Madagascar primates had an abnormally low spontaneous mutation rate.

D. Detection of Endogenous Viral Sequences in Man.

The results to date using representative cDNAs from virus isolates to detect human endogenous sequences have been negative. There are two useful approaches for obtaining more appropriate viral probes. The first one is to determine whether there are regions of the viral genome that are more conserved

than average and whether these regions taken from available viruses will suffice. Data with the strong stop sequence from CPC-1 suggests that strong stop sequences are not more conserved than average. Further dissection of the genome will proceed when we have cloned unintegrated viral DNAs. The second approach is to obtain viral sequences from a closer relative to man. To this end we are in the process of cloning the type-C virus-related sequences from chimpanzee, the closest relative of man for which we can detect related sequences using presently available viral cDNAs.

C. Organization of Unintegrated and Integrated Viral DNA Sequences and Their Relationship to Biological Activity.

Analysis of the unintegrated DNA of baboon endogenous virus (M7) showed two molecular forms. The minor form consisted of two covalently closed circular DNA species of approximately 9.0 Kb and differing from each by about 0.5-0.6 Kb. The smaller of the two was in 2-3 fold excess over the larger species. The circular nature of these two DNA species was substantiated by restriction enzyme analyses. The major form of unintegrated M7 DNA consisted of a single species of linear DNA corresponding to 9.0 Kb and had a 0.6 Kb direct terminal repeat on each end. The linear DNA was biologically active when transfected onto dog cell, bat cell or human cell recipients and the virus produced exhibited properties similar to the parent M7.

A physical map of the infectious linear DNA was constructed by digesting the DNA with different restriction enzymes and locating their sites of recognition on the viral DNA. The enzymes include one no cut enzyme, one enzyme that cuts only in the terminal repeats, and three enzymes that make one cut. The order of these fragments was determined by double digestion and the viral DNA was oriented with respect to the 3' and 5' end of viral RNA. The integrity of the terminal repeats in the unintegrated linear viral DNA as well as in the integrated DNA appears to be essential for biological activity. Removal of part of the terminal repeats by digestion with Xho I which cuts only in the terminal repeats abolished biological activity. However, the untreated DNA as well as the DNA digested with the no cut enzyme retained biological activity. We are using recombinant DNA techniques to clone unintegrated DNA which should allow the determination of the minimum nucleotide sequence necessary for biological activity.

The specific site of the infectious viral DNA used in integration was investigated by restriction analyses of infectious high molecular weight DNA. No detectable permutations of viral DNA sequences compared to the linear map and no significant deletions or substitutions in the viral DNA were found. The sequences in the end region of the terminal repeats of viral DNA were used in fusing with cellular DNA sequences. Analyses of cellular DNA with respect to its sites of integration by restriction enzyme showed that the fusion fragments (consisting of cellular and viral sequences) were unique

for each clone and the size of the fragments was different in different clones. These results show that several sites in the cell DNA can accommodate viral DNAs.

Restriction analysis of the multiple copies of the M7-related sequences in baboon DNA shows that their organization is surprisingly simple. The cellular DNA sequences flanking the endogenous viral DNA sequences are reiterated indicating common sites of residence in cell DNA. The viral sequences appear to be non-permuted and non-tandem. There are also no significant insertions or deletions in the viral DNA except possibly in the terminal repeats. In spite of the similarity of the endogenous sequences to the M7 sequences in infected cells, the endogenous sequences were not biologically active. Further analyses of the organization of the endogenous sequences will be pursued using specific probes prepared from cloned viral DNA. In addition, we plan to use the green monkey genomic library provided by Dr. Maxine Singer to isolate the three classes of viral sequences from a single species and to characterize the flanking sequences.

b. Transcription and Processing of Virus-specific RNAs in CPC-1 Infected Cells.

The expression of primate retroviruses in productively and nonproductively infected cells is being investigated employing RNA blotting procedures. The combination of a poly (A) selection procedure applied to the RNA molecules with the use of a probe specific to the 5' end of the virus ensures that any alterations in the size of these molecules must be the result of post-transcriptional modifications (splicing). The application of this analytic procedure to CPC-1 infected human lung carcinoma cells (A549 cells) revealed two virus-specific RNAs (8.60 Kb and 2.95 Kb) which likely represent the genome-length "gag" and "pol" messages and the spliced "env" message, respectively. Interestingly, following the treatment of these cells with DMSO (a chemical known to modify cellular transcriptional controls) an additional RNA species of 4.5-4.6 Kb in length has been detected which hybridizes to the virus-specific probe. We are presently investigating the origin of this novel RNA, i.e., does it represent an intermediate in the splicing of viral RNAs or the derepression of cellular mRNA which contains sequences related to those found to be common among the retroviruses.

Significance to Biomedical Research and the Program of the Institute:

Our evolutionary data strengths the hypothesis that the three classes of endogenous Old World primate viruses have been genetically transmitted since the early stages of primate evolution. The corollary that such sequences should exist in man is thereby also strengthened. Although actual detection of these sequences still eludes us, the data guides us in the next stages of the search. The first approach will try to dissect the viral genome to define especially conserved regions in the available viruses which might allow detection of related human sequences. The dissection of the viral

genome may also provide more general information on viral evolution or on the evolution of cellular repeated sequences. The second approach is to isolate the virus-related sequences from close relatives of man by molecular cloning techniques.

The quantitative differences in evolutionary patterns for the 3 classes of viruses demonstrates that they have been subject to differing evolutionary pressures and therefore do not play a totally neutral role in the cells. The molecular cloning of viral sequences and their restriction mapping provides us with powerful tools to study their biological activity at the molecular and cellular levels. The results to date suggests that the organization of the endogenous primate viral sequences is simpler than for the murine and avian cases. This probably occurs because the possibility of infection of primate cells with closely related viruses is quite limited.

Proposed Course:

The existence of endogenous viruses indicates that similar viral genomes or related sequences may exist in human cells. Since we would ultimately like to characterize such human viral sequences we have focused on the viruses isolated from man's closest relatives, the Old World monkeys. Irrespective of their direct relationship to a putative human virus, these viruses compose a useful system in which to study the general characteristics of endogenous viruses. The primate system parallels the avian and rodent systems but has somewhat different characteristics from the other two systems which may be related to the fact that all the present primate viruses isolates are xenotropic. For these reasons this project will be continued and extended along the lines already described.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05107-02-LVC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Phosphoproteins and Protein Kinases of Mammalian Retroviruses: Interactions with Viral Genomes and Cellular Cytoskeleton

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Sen	Visiting Associate	LVC	NCI
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COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

All replicating retroviruses studied to date contain one major structural phosphoprotein which binds preferentially to the homologous viral RNA genome in-vitro. The specificity and evolution and the effects of phosphorylation on this protein-nucleic acid interaction has been studied using a wide variety of related retroviruses. A number of replication-defective sarcoma viruses have been examined for specific phosphoproteins and protein kinase molecules in pseudotype virion particles containing each sarcoma virus. RNA-phosphoprotein binding studies with the Rauscher MuLV p12 subpopulation, separated on the basis of their levels of phosphorylation, suggested that the level of phosphorylation of these molecules regulate the extent and not the specificity of binding. The viral RNA-binding of the phosphoproteins of replicating retroviruses of endogenous mammalian origin represents a specific nucleoprotein interaction which is evolving with the species. This is regulated by phosphorylation and might be involved in the genetic as well as horizontal transmission of these oncogenic viruses.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

All replicating retroviruses studied to date contains one major structural phosphoprotein which binds preferentially to the homologous viral RNA genome in-vitro. The specificity and evolution and the effects of phosphorylation on this protein-nucleic acid interaction has been studied using a wide variety of related retroviruses. A number of replication-defective sarcoma viruses have been examined for specific phosphoproteins and protein kinase molecules in pseudotype virion particles containing each sarcoma virus. At least in the case of murine sarcoma virus, a sarcoma virus-specific low molecule weight protein kinase activity binds to cytoskeletal proteins. Replication-competent, nontransforming "helper" retroviruses and replication-defective, transforming "sarcoma" viruses are derived from endogenous cellular genes. Studies on the biological activities of the specific phosphoproteins and protein kinases of these viruses are aimed at furthering our understanding of the regulatory roles of such viral gene products in the origin and transmission of oncogenic RNA tumor viruses.

Methods Employed:

Molecular studies on purified, biologically active phosphoprotein molecules and protein kinases of retroviruses have been pursued to obtain information regarding the origin and evolution of these molecules, the macromolecules they interact with, and the possible role of phosphorylation in these processes. Advanced biochemical and molecular biological techniques were used.

Major Findings:

The early demonstration of preferential binding of p12 phosphoprotein molecules to the homologous viral RNA genome of the infectious type-C viruses of mouse, cats, and in the infectious primate (woolley monkey and gibbon ape) leukemia viruses of endogenous murine origin suggested a regulatory role of this binding in the in vivo life cycle of the replicating type-C viruses. Experiments designed to locate the binding sites on the viral RNA genome detected the presence of a strong cluster near the 3' polyadenylated terminus (presumably within the common sequence) of the viral genomic RNA subunit. The use of uv-irradiation for in situ crosslinking, suggested that a small number of structural phosphoprotein molecules was packaged in close association with the viral genome. RNA-phosphoprotein binding studies with the Rauscher MuLV p12 subpopulation, separated on the basis of their levels of phosphorylation, suggested that the level of phosphorylation of these molecules regulate the extent and not the specificity of binding. A systematic analysis of endogenous viruses of closely related primates showed that the genomic binding sites and the virion structural phosphoprotein molecules

shared considerable levels of homology. Studies on the recent isolates of endogenous primate retroviruses showed that each contains one major class of structural phosphoprotein and in each case these molecules are bound to the viral RNA. The viral RNA-binding of the phosphoproteins of replicating retroviruses of endogenous mammalian origin represents a specific nucleoprotein interaction which is evolving with the species. This is regulated by phosphorylation and might be involved in the genetic as well as horizontal transmission of these oncogenic viruses.

The fibroblast-transforming sarcoma viruses of mammals share extensive homologies with replication-competent helper viruses derived from the same species. An extensive survey of different sarcoma virus pseudotypes with different helper viruses, grown in different cells, has been undertaken to search for specific phosphoprotein molecules in pseudotype virions containing specific sarcoma viruses. These studies indicated that each mammalian sarcoma virus pseudotype contains at least one major size class of phosphoproteins which are independent of the helper virus used and also of the cell lines where the viruses were propagated. Recently, using a high titer mouse sarcoma virus (MSV) stock, we detected a class of low molecular weight protein kinase activity and phosphoprotein molecules migrating at 15-16K daltons, neither can be found in the nontransforming helper alone. This MSV-specific activity binds to actin in vitro and also inhibits the polymerization of microtubular proteins in an in vitro assay. It is a Mg^{2+}-dependent enzyme which does not require cyclic AMP for its activity. It is an internal protein of MSV virions since detergent disruption is required for its release from virions. At least in one temperature sensitive transformation mutant of MSV, this low molecular weight kinase which binds to actin is thermolabile.

Significance to Biomedical Research and the Program of the Institute:

The phosphoproteins and protein kinase activities of murine leukemia and sarcoma viruses by virtue of their ability to interact with viral and/or cellular nucleic acids and cellular cytoskeletal proteins represent candidate regulatory products which might play important roles in viral replication and host cell alterations. Studies on the effects of binding of phosphoprotein molecules to viral RNA on the template activity of the genome might offer new insights in the regulation of reproduction of replicating retroviruses. An examination of the manner of interactions of the MSV kinase with cytoskeletal proteins and other cellular substrates will likely extend our fundamental understanding of the molecular mechanisms underlying cell transformation.

Proposed Course:

This project has been completed.

Publications:

Sen, A., Halverson, D.O., Rapp, U.R. and Todaro, G.J.: Sarcoma virus-specific phosphoproteins are packaged in "rescued" type-C virions. Virology, 92:245-251, 1979.

Sen, A., and Todaro, G. J.: A murine sarcoma virus-associated protein kinase: Interaction with actin and microtubular protein. Cell 17:347-356, 1979.

Sen, A., Todaro, G. J., Blair, D. G., and Robey, W. G.: Thermolabile protein kinase molecules in a temperature sensitive murine sarcoma virus pseudotype. Proc. Natl. Acad. Sci. (USA) 76:3617-3621, 1979.

Keski-Oja, J., Sen, A. and Todaro, G. J.: Direct association of fibronectin and actin molecules in vitro. J. Cell Biol. (in press), 1980.

Keski-Oja, J., Sen, A. and Todaro, G. J.: Direct association of fibronectin and actin molecules in vitro. Cell (in press), 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05108-02-LVC

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Growth Factors Released by Transformed Cells: A Possible Mechanism Responsible for the Transformed Phenotype

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. De Larco	Expert	LVC	NCI
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	U. R. Rapp	Visiting Scientist	LVC	NCI
	J. Keski-Oja	Visiting Fellow	LVC	NCI
	H. Marquardt	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A hormone-like cell growth factor (Sarcoma Growth Factor - SGF) was detected, isolated, and characterized. SGF effected a reversible malignant phenotypic change in cultured cells. These studies were done to determine how these substances biochemically bring about the morphological phenotype of transformed cells. Serum-free conditioned media was searched for growth factors from MSV-transformed 3T3 cells. An acid soluble peptide extract stimulated ^{3}H-thymidine incorporation into the DNA of serum depleted cells, competed with radiolabeled EGF for plasma membrane receptors, and stimulated anchorage independent growth of untransformed cells in soft agar. Comparison of SGF and EGF (Epidermal Growth Factor) showed that while they share some common properties they are distinctly different. Both interact with the EGF membrane receptor system, are heat stable peptides requiring disulfide bonds for activity, and are active in nanogram quantities. They differ in that SGF allows soft-agar growth where EGF will not; SGF is produced by MSV-transformed cells whereas EGF is not. Antibodies directed against EGF do not recognize SGF, and their isoelectric points are distinctly different, EGF being 4.4 and SGF being 6.8.

Project Description

Objectives:

To purify and sequence the major classes of growth factors released by murine sarcoma virus transformed fibroblasts. Sequences of these purified peptides will be compared with each other as well as to sequences of well characterized peptide hormones and growth factors to determine if any homologies exist. Specifically, radioimmune assays will be developed for these peptides to determine if either they or peptides related to them are expressed either during development or by certain tissues under normal physiological conditions; the level of this type of peptide expression in tumors will be examined and compared with those of the homologous normal tissues; the interaction of these growth factors with normal cells will be studied to determine the sequence of events required for phenotypic transformation in contrast with those events already described concerning known growth factors (such as EGF).

Methods Employed:

Transformed cells grown in-vitro show a multiplicity of changes in comparison with their untransformed counterparts. They express changes in their growth pattern. While normal cells tend to grow in flat, regular geometric patterns, transformants tend to criss-cross over one another and form multiple layers containing cells of heterogeneous size and shape. Other major alterations are found in their growth characteristics. Nontransformed cells require attachment to a substrate for proliferation, a property called "anchorage dependence". Transformed cells have decreased adhesion to the substrate and may have acquired the ability to overcome anchorage dependence. Growth in semisolid media or suspension serves also as an indicator of anchorage independence and is considered a good criterion of transformation. Non-transformed cells usually require protein factors from serum to continue proliferation and growth; transformation is characteristically accompanied by a reduction in the requirement for serum factors. Our previous observations have shown that murine sarcoma virus (MSV) transformed cells have a decrease in measurable plasma membrane receptors for epidermal growth factor (EGF), a potent mitogen. This decrease was specific in that MSV-transformation did not affect the level of expressed plasma membrane receptors for several other ligands [i.e., insulin, multiplication stimulating activity (MSA), viral glycoprotein, or concanavalin A] nor did transformation by DNA tumor viruses cause a decrease in the level of EGF receptors. Consistent with these observations, we made the hypothesis that MSV-transformation causes the transformed cell to produce and release a growth factor that was either EGF or an EGF-like peptide capable of binding to the EGF receptor system and thus stimulating cell growth and decreasing the serum requirement as well as acting as an approximal effecter of transformation. The rationale

for the studies was to determine if MSV-transformed cells produced an EGF-like factor or factors, and if so, did they have the properties capable of explaining, on a biochemical basis, the morphological phenotype of transformed cells.

Major Findings:

A. Detection Sarcoma Growth Factor

Serum-free conditioned media was used as the starting material in the search for growth factors from MSV-transformed 3T3 cells. An acid soluble peptide extract was found to stimulate ^{3}H-thymidine incorporation into the DNA of serum depleted cells, compete with radiolabeled EGF for plasma membrane receptors, and stimulate anchorage independent growth of untransformed cells in soft agar. This activity exceeded that of whole serum or mouse submaxillary gland EGF though the specific activity suggested the EGF-competing activity was only a minor component in the extract. When the extract was tested on normal rat kidney cells (soft-agar assay), the cells responded by forming colonies. The size and percent of the colonies was dose dependent (Table 1). Several large colonies were picked and allowed to grow in monolayers in media containing 10% calf serum. These cells expressed a "normal" morphology in monolayers and when placed in agar containing media with 10% calf serum no colonies were formed; however, when they were placed in agar in the presence of the extract, the cells produced uniform large colonies. This biological activity was acid and heat stable and required disulfide bonds for biological activity. A chromatographic profile from a P-60 column showed 3 major classes of EGF-competing activities. The largest and most

Table 1

Effects of Peptides Present in MSV-conditioned Media on the Growth of Fibroblastic NRK Cells in Soft Agar

Substance and Concentration ng/ml	Distribution by Colony Size, Number <0.032 mm	0.032-0.064 mm	0.065-0.130 mm	>0.130 mm
SGF				
56	1	9	60	30
14	27	42	23	3
3.5	32	61	7	0
EGF				
100	100	0	0	0
NONE				
	100	0	0	0

heterogeneous had an apparent molecular weight of 20,000-27,000. The other 2 eluted with apparent molecular weights of 10,500 and 6,000.

B. Concentration of Sarcoma Growth Factor

The activity that stimulated cells to form colonies in soft agar eluted with a peak of EGF competing activity. The peak at 10,500 had more soft-agar colony-forming activity (5×10^5 units/mg of protein) per unit of EGF-competing activity than any other peak. Its specific colony-forming activity peak was approximately 200 times greater than that in the original medium. A low molecular weight peak contained 25×10^5 units per mg. Peaks of activity responsible for stimulating thymidine incorporation coeluted with the EGF competing activities. An intermediate sized peptide (pool II) was chosen for further study because it had the highest specific activity and gave rise to larger soft agar colonies. It was named Sarcoma Growth Factor (SGF). The activities of SGF (stimulation of thymidine incorporation, EGF competition, or soft agar growth stimulation), when purified by DEAE-cellulose or CM-cellulose chromatography (De Larco and Todaro, J. Cell Physiol. in press; De Larco et al., in press) were found to coelute from these columns. This suggested that SGF exerted its biological activity via the cell membrane EGF receptors. To test this, clones from 3T3 cells were used, specifically: An EGF receptor-less clone (NR6/6) (Press and Herschman, Proc. Natl. Acad. Sci. (USA) 74:3918, 1977) and the parental clone (3T3/8) which retains functional EGF receptors. While 3T3/8 is responsive to the mitogenic action of EGF, the selected clone, NR6/6 lacks detectable EGF receptors, and is not responsive. The 3T3/8 cells and the NR6/6 clone were tested in parallel for their response to calf serum and to purified growth factors, including EGF and SGF. The parental clone, 3T3/8, responded to all of the growth factors tested, as determined by ^{3}H-thymidine incorporation in DNA. The NR6/6 clone, on the other hand, responded to all of the growth factors except EGF and SGF (ion exchange purified). DNA syntheses was stimulated in both clones when they were treated with calf serum, fibroblast growth factor (FGF), multiplication stimulating activity (MSA), or the MSA-like peptide activity released by a human fibrosarcoma (De Larco and Todaro, Nature 272: 256, 1978). In the soft agar assay, the 3T3/8 cells responded readily by developing a high percentage of large colonies when treated with SGF, whereas the EGF receptor-less clone, NR6/6 did not respond at all. These results led to the belief that available EGF receptors are required for SGF to exert its biologic effects in mouse 3T3 cells. If SGF acts through the EGF receptors, direct binding should be demonstrated if the protein is pure enough. A portion of the DEAE-cellulose purified fraction was therefore iodinated and tested for specific binding to cell surface receptors. A confluent culture of a cell line developed from a human vulva carcinoma, A431, with an unusually large number of surface EGF receptors was used for binding. Two unlabeled proteins were tested for their ability to compete with the ^{125}I-SGF for binding to these cells' plasma membrane receptors as well as mouse submaxillary gland EGF and SGF from Pool II. Although SGF had a much lower specific activity, both competed to the same extent with the binding of ^{125}I-SGF. The amount of ^{125}I-SGF specifically bound to these cells was more than six-

fold the nonspecific binding; approximately 1% of the input counts bound in a competable fashion. Competitions to the ^{125}I-SGF binding were run using unlabeled SGF (pool II) and unlabeled EGF. The slopes of these competitions were parallel, and the amount of unlabeled peptides required for a 50% competition were 0.57 ng for EGF and 480 ng for the Pool II SGF.

C. Purification of Sarcoma Growth Factor

These observations suggested formaldehyde fixed A431 might act as an "affinity phase" to purify ^{125}I-labeled SGF. The cells were fixed in formaldehyde solution and the radiolabeled peptide pool was incubated for 1 hour with the fixed cells. The unbound labeled peptides were removed and the cells washed twice with binding buffer (De Larco and Todaro, Cell 8:365, 1976) to remove any remaining unbound radiolabeled peptides. The "bound" peptides were then eluted by washing the fixed monolayers with 0.1% acetic acid and concentrated by lyophilizing. Using the DEAE-cellulose SGF for labeling the percent of the input bound rose from 0.97% for the uncycled material to 16.% for the material after the first cycle and to 29.7 % for the material after the second cycle. The same procedure was used on the mixture of iodinated peptides from the peak fraction of the Pool II. The specific binding rose from 0.12% of the input counts for the uncycled pool to 23.9% after the second cycle (Table 2). The isoelectric point of radiolabeled uncycled peptides was compared with that of cell cycled peptides for both EGF, a pure peptide to start with, and Bio-Gel P-60 Pool II, a peptide mixture containing the SGF. Isoelectric focusing of the ^{125}I-labeled EGF preparation prior to purification using fixed A431 cells gave two sharp peaks: The smaller peak with a pI of 3.8 and the larger peak with a pI of 4.4. The labeled EGF that had been bound to and eluted from fixed cells gave a single sharp peak upon isoelectric focusing with a pI of 4.4. This value is consistent with that previously published for EGF. In contrast, isoelectric focusing of iodinated crude SGF gave a heterodisperse profile with the majority of the labeled

Table 2

Purification of ^{125}I-SGF using Binding and Elution from Fixed A431 Cells

Material	Specific Binding Percent of Input Nonspecific Binding	dpm Bound
Bio-Gel P60 Pool II	0.18	0.12
Bound and Eluted Through One Cycle	5.6	2.8
Bound and Eluted Through Two Cycles	18.7	23.9

material having acidic pI values. The radiolabeled peptide(s) from crude SGF bound to and eluted from the fixed A431 cells, however, gave a sharp peak upon isoelectric focusing with a pI of between 6.8 and 7.0.

SGF and EGF share common properties yet are distinctly different. They are similar in that they both interact with the EGF receptor system, are heat stable peptides requiring disulfide bonds for activity, and are active in nanogram quantities. They differ in that SGF allows soft-agar growth where EGF will not; SGF is produced by MSV-transformed cells whereas EGF is not. Antibodies directed against EGF do not recognize SGF, and their isoelectric points are distinctly different, EGF being 4.4 and SGF being 6.8.

In a temperature sensitive mutant of MSV the production of SGF is temperature sensitive whereas the isolated SGF from conditioned media collected at a permissive temperature did not appear to be temperature sensitive under the conditions assayed (De Larco et al., submitted for publication).

Significance to Biomedical Research and the Program of the Institute:

This work is of significance and high priority because at a basic research level it seeks to define the mechanisms for the control of cell reproduction and the phenotypic responses to malignant transformation. At the applied level, it aims to detect and characterize growth factors that are closely related to the control of cell growth in normal and cancer tissues. These goals are in the main stream and forefront of the mission of the NCI and will produce results that will be applicable to patient.

Proposed Course:

This project will continue in two phases: (a) Continue the characterization of Grwoth Factors, like SGF, to better understand the relationship between the phenotypic and genotypic aspects of malignancy, and the principles governing each. (b) To apply the same principles and technology to the isolation and characterization of factors, like SGF, isolated from human tumor cells. These human factors will be used to detect their related producing genes and to study the relationship of such genes to the process of malignant transformation.

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Todaro, G.J., Callahan, R., Rapp, U.R. and De Larco, J.E.: Genetic transmission of retroviral genes and cellular oncogenes. Phil. Trans. Royal Soc. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05124-03-LVC

PERIOD COVERED
September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)

Modulation of Gene Expression in Primate Placenta

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	L. K. Donner	Visiting Scientist	LVC, NCI
	R. I. Huot,	Biologist	LVC, NCI
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COOPERATING UNITS (if any)

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LAB/BRANCH Laboratory of Viral Carcinogenesis

SECTION Viral Pathology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The gene regulation relating to malignancy that was studied included primate retrovirus expression, and modulation of human chorionic gonadotropin, and placental alkaline phosphatase. The effort to induce novel primate retroviral expression utilizes transfection by early region SV 40 DNA of cell strains to obtain permanent indicator lines to overcome host range restriction and for use in retroviral induction, and use of Rhesus monkey and human trophoblast in cell and organ culture as targets for retroviral isolation. Improved methods have been developed for growth of human placenta in organ culture and for isolation of trophoblast cells for growth in monolayer culture.

Rhesus placental tissue, but not other Rhesus fetal organs, contain MAC-1 p26 expression, and this persists in trophoblast cell and organ culture. A Rhesus trophoblast cell stain transfected by SV 40 Gene A displays prolonged growth in cell culture, and expression of placental alkaline phosphatase activity, type IV collagen, laminin, fibronectin and by EM contain desmosomes, but no p26 activity.

Project Description

Objectives

Understanding the mechanism of expression of genes associated with malignancy represents both a potential diagnostic approach and a therapeutic monitor for cancer control. Our laboratory has selected two broad categories of gene products - retrovirus expression in primate placenta and certain differentiated functions in placental tissue - whose expression are closely related to malignancy. We have focused on gene products either implicated as present in human cancer (retroviral expression) or clearly related to human cancer, for example, human chorionic gonadotropic (HCG) and placental alkaline phosphatase (PAP). The objectives have been i) to compare the modulation of HCG and PAP in human choriocarcinomas and normal human trophoblast in cell and organ culture, ii) to establish that there is a tissue-specific preference for expression of endogenous type C virus in rhesus placenta, iii) to enhance the efficiency of expression and isolation of endogenous Rhesus type C virus from placental tissue *in vitro*, and iv) to apply principles established in the Rhesus system to primate species from which retroviruses have not been isolated.

Methods Employed

Rhesus retroviral expression is scored by radioimmunoassay of MAC-1 p26, and cell and/or organ culture supernates are screened by DNA polymerase assay, using rAdT synthetic templates of clarified pellets. An RIA for HCG secretion as a biochemical marker of trophoblast function *in vitro*, and indirect immunofluorescent detection of type IV collagen, laminin, and fibronectin are additional assay procedures.

Major Findings

Optimal methods have been developed for growth of human placenta in organ culture and for isolation of trophoblast cells for growth in monolayer culture. HCG expression in human placental organ culture is highest under conditions of Gelfoam support, incubation of 95% air and 5% CO_2 in a chamber on a platform rocker, and use of CMRL 1066 medium with FCS, insulin, hydrocortisone, and retinal acetate. The kinetics of HCG secretion are in the manner of a bell shaped curve with peak levels around day 6 in organ culture. Addition of theophylline and dibutyryl cyclic AMP elevated HCG levels by about 4-fold over untreated controls. The amount of HCG expression with this improved organ culture system were approximately ten thousand times higher on a per gram basis than those obtained with term placenta in conventional explant culture.

Human choriocarcinoma and normal human trophoblast are maintained in cell and organ culture, and modulation of chorionic gonadotropin, and placental alkaline phosphate are being examined under the influence of cyclic

nucleotides and metabolic inhibitors. THe relationship between inhibition of DNA synthesis and the stimulation of chorionic gonadotropin (HCG) in cultured human choriocarcinoma cells was extended from the BeWo line (Cancer Res. 36: 4570-4576, 1976) and to JAR line (Cancer Res. 39: 1952-1957, 1979) to normal first trimester human placenta in organ culture. Four compounds previously shown to elevate the levels of HCG secretion by JAr cells or other choriocarcinoma cells were employed: epidermal growth factor (EGF), methotrexate (MTX), 5'-fluoro 2'-deoxyuridine (FUdR), and actinomycin D (AMD). A partial inhibition of DNA synthesis permitted enhanced HCG production in JAr cells under some conditions but not in others while a complete cessation of DNA synthesis was inhibitory. This indicated that an additional mechanism other than the inhibition of DNA synthesis was necessary for the enhanced production of HCG by the JAr cells in the presence of MTX. The culture medium employed profoundly influenced the ability of MTX to stimulate HCG secretion in JAr cells. Of the four modulation agents above, placental cultures responded only to FUdR and AMD suggesting that the regulation of HCG synthesis differed in the JAr line and in the normal placental cultures. EGF only modestly increased HCG production in the JAr cells (about two-fold) and did not enhance HCG synthsis in the placental cultures despite the presence of receptors for EGF on both JAr cells and normal placental tissue in organ culture.

The greater utilization of the salvage pathways in nucleotide synthesis by the placental cultures could lead to a divergent effect of MTX on cellular processes, such as transcription, thus resulting in an increased HCG production only in the JAr cells. The stimulation of HCG production by AMD, and possibly FUdR, in the placental cultures might be the result of post-transcriptional modification of HCG synthesis. The further stimulation of HCG secretion after the placental organ cultures had been exposed to both dbcAMP and AMD suggested that AMDS, and perhaps the other drugs examined, elevated HCG production by a superinduction mechanism. A similar effect may have caused the observed stimulation of HCG secretion in the JAr cells by MTX and FUdR.

Cell strains from various sea mammals have been sucessfully transfected (T antigen positive) by the early region of SV 40 DNA to serve as additional indicator cell lines to overcome retroviral host range restrictions. These six sea mammal cell strains grow rapidly and vigorously, and will be suitable for use as indicator cell lines. Karyotopic analysis indicates that 4 of these T-ag positive lines which reached senescence at approximate passage 50 were diploid, whereas the remaining 2 were hyperdiploid and are continuing to grow well after over 70 passages. Consequently, T-ag positivity does not necessarily lead to immortality in cell culture. However, the paired untransfected parental strains displayed much less longevity in cell culture, smaller or no colony growth in soft agar, and a lower plating efficiency than the transfected daughter sea mammal lines. Prolonged cocultivation of these sea mammal strains themselves with a variety of heterologous species has not yielded endogenous retroviral expression.

Similarly, a Rhesus trophoblast cell strain has been established after transfection by SV 40 Gene A which is currently at passage 14, and expresses placental alkaline phosphatase activity, type IV collagen, laminin, fibronectin, but no MAC-1 p26 production, or supernatant DNA polymerase expression after cocultivation with cell lines from heterologous species. In contrast, freshly isolated Rhesus trophoblasts expressed MAC-1 p26 at levels equivalent to the placental tissue from which they were isolated, and p26 antigen expression was maintained during placental organ culture after 12 days in culture.

Endogenous retroviruses have been isolated by cocultivation of primate tissues with indicator cell lines from heterologous species that are permissive for viral replication. Although it is well established that DNA sequences related to endogenous baboon (M-7) and Rhesus (MAC-1) type C viruses can be detected in the DNA of all Old World monkey, baboons, Rhesus, and colobus monkeys are the only Old World primates that have yielded complete infectious virus in cocultivation experiments. Moreover, the frequency of isolation of such viruses varies between baboon and rhesus: while baboon retrovirus has been obtained from many tissues and cultured cells, Rhesus type C virus has only been obtained on two occasions out of numerous attempts. Thus, it appears that different species of primates yield endogenous viruses either frequently, rarely, or not at all.

The expression of endogenous primate retroviral genes may also vary in different tissues. For example, ultrastructural studies have suggested that type C viral particles can be detected in primate placental tissue but not in other fetal organs. While a tissue-specific preference for type C viral expression may not be of particular importance in species from which viruses are frequently obtained, among primates which yield retrovirus only rarely, this could be a major variable. To examine this question, attempts have been made to study type C viral expression in placental organ and cell cultures and to establish conditions whereby endogenous retroviruses might be more readily isolated from primate species.

The radioimmunoassay for MAC-1 p26 was used to score antigen expression in Rhesus placenta and other fetal organs obtained at various times of gestation. Antigen expression was detected in 14 out of 14 placental specimens but not in fetal heart, lung, spleen, liver, kidney, intestines, skeletal muscle, testes, and endometrial scrapings. The levels of antigen detected in placenta ranged between 6 and 218 ng/mg protein with no apparent correlation between quantity of antigen and gestational age or parity. A 10-fold higher level of antigen expression was detected at the decidual surface than at the amniotic surface of the placental specimens. Thus, even within the placenta, there was a preferred site for endogenous retoviral antigen expression. Rhesus trophoblast and organ cultures were cocultivated with appropriate heterologous indicator cells lines. In seven

attempts to isolate endogenous type C viruses by cocultivation techniques, foamy virus was obtained from 4 specimens, no virus from one explanted placenta after 3 months of cocultivation, and two placental specimens yielded retrovirus of probable endogenous origin.

Significance to Biomedical Research and the Program of the Institute

The relevance of the retroviral animal model to human cancer can be tested when a bona fide retrovirus of human origin is growing in cell culture in adequate yield. Only then can appropiate detection techniques, such as radioimmune precipitation assays for certain viral polypeptides, be developed to correlate retroviral expression with neoplastic growth in man. This project is in part devoted to this aim by using the Rhesus placenta as a model system. As factors which influence retroviral expression in what may be a physiological manner in Rhesus placenta become evident, they will be adapted toward retroviral induction in higher primate species, including man, from which retroviruses have not yet been isolated.

Knowledge of mechanism of modulation of expression of biochemical markers of malignancy, such as HCG and PAP, is essential for the markers to be useful clinically. These two markers of human cancer can be easily studied in normal and malignant human placental tissue in cell and organ culture.

Proposed Course

Attempts will be made to utilize other assays for MAC-1 viral gene expression in the rhesus trophoblast system. Both purified MAC-1 gp60 and antisera are available and will be employed to develop an IRA for the MAC-1 envelope glycoprotein. This will permit an analysis of MAC-1 viral gene products which may be non-coordinately expressed. Transcription of viral genes will also be monitored using established reverse-Southern blot procedures, using RNA from organ cultures and cell strains. The results from these tests should enable the evaluation of potential viral inducing agents as correlated with assays for trophoblast gene function.

Publications

Huot, R., Foidart, J.-M, Stromberg, K.: Effects of culture condition on the synthesis of human chorionic gonadotropin by placental organ cultures. In Vitro 15: 497-502, 1979

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Stromberg, K., Donner, L., Yee, C. L. Howley, P. M.: Growth potential of sheep and sea mammal cells transformed by SV 40 early region DNA. Proc. Soc. Expt. Biol. Med., In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05143-01-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies on Burkitt's Lymphoma in the Western Hemisphere

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Information was collected on 256 pathologically confirmed cases of Burkitt's lymphoma (BL) diagnosed in the western hemisphere. The curability of BL was confirmed, particularly in patients with limited disease. Target organs appeared to be related to age, suggesting a predilection of the disease for rapidly dividing cells. Laboratory and epidemiologic studies provided evidence for environmental as well as genetically determined predisposing factors. Patients with Epstein-Barr virus (EBV) associated tumors appeared to have a longer survival rate than those with non-EBV associated tumors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The purpose of this project is to study the disease pattern of Burkitt's lymphoma (BL) and its association with Epstein-Barr virus (EBV) in a non-endemic area, the United States, where an important cofactor in African BL, malaria, is not present.

Methods Employed:

In the past year, 150 cases were collected from the Clinical Center at NIH, from the literature, and from personal contact with various physicians from different parts of the country. The patients' physicians were contacted for clinical information, diagnostic blocks, and sera. The diagnostic blocks were sent to Dr. Berard (NIH) for review and confirmation of the diagnosis. The sera obtained from these cases were tested for antibody titers to the EBV viral capsid antigen (VCA) and early antigen (EA). The data from 256 BL patients were then analyzed for various racial, geographical and epidemiological patterns and compared to the African patterns.

Major Findings:

The major characteristics of American BL patients tended to be male predominance, a young age group involvement (4-12 yrs.), and a higher incidence in Caucasians. More recently older patients have been frequently reported, perhaps due to better awareness of the disease on the part of non-pediatricians. In contrast to the earlier report showing a paucity of patients in the high altitudes, cases were obtained from all parts of the country. Positive cancer history was obtained in first degree relatives of 18 of the 152 cases on whom detailed pedigrees could be obtained. Unlike African cases, where the jaw is the most common presentation, abdominal viscera were affected most often, the most outstanding being the ileum. In American cases, cervical lymph nodes were also among the most common organs to be involved. Better median survival rates for Stage I and II cases was observed than for patients with Stage IV disease. No relapse was observed in American cases beyond 2 years, whereas 12 late relapses were observed in a series of 447 African patients. The survival seemed to be prolonged in patients with elevated VCA antibody titers.

Significance to Biomedical Research and the Program of the Institute:

Burkitt's lymphoma (BL) has been one of the tumors with the most prominent epidemiologic and laboratory evidence indicating a viral etiology. Two major cofactors implicated in the cause of BL are malaria and the Epstein-Barr virus. Studies on BL in an area where malaria is absent may help to clarify the role of EBV in the pathogenesis of this disease.

Proposed Course:

In addition to continuing to identify new BL patients and observing of patients already entered into the American BL Registry, we intend to analyze in detail records of those patients first diagnosed as BL and then reclassified as malignant lymphoma, undifferentiated, non-BL, to determine if they have a different relationship to EBV and/or a different clinical pattern.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05135-01-LVC

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Murine and Human Monoclonal Antibodies Detect Human Mammary Tumor Associated Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION

Tumor Virus Detection Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed methodology for the in-vitro synthesis of human monoclonal antibodies from human-mouse hybridoma cell lines. These human-mouse hybridomas have been obtained by the fusion of human lymphocytes (from sections of lymph nodes from mastectomy patients) with nonproducer murine myeloma cells. Employing solid phase and liquid RIAs, we have shown that antibodies produced by these hybridoma cell lines are either human IgG or human IgM. The levels of immunoglobulin production by these human-mouse hybridomas are comparable to those of mouse-mouse or rat-mouse hybrids, synthesizing between 0.1 and 20 ug of immunoglobulin per ml tissue culture fluid; the duration of immunoglobulin synthesis ranged to 130 days post fusion. Some of the human immunoglobulins synthesized showed reactivity by the immunoperoxidase technique with human mammary tumors (including the tumor of the patient from which the segments of nodes were obtained). These same antibodies did not react with normal mammary epithelium found in the same breast; the specificity of this reactivity is currently under investigation.

PHS-6040
(Rev. 10-76)

Project Description

OBJECTIVES: To generate monoclonal antibodies that identify tumor-associated or tumor-specific antigenic determinants of murine and human mammary carcinoma cells. Specific aims include:

1. The use of monoclonal antibodies for the identification of antigenic determinants (viral and nonviral) associated with, or specific for, murine mammary carcinoma cells. Monoclonal antibodies will also be employed in therapy studies of murine mammary tumors.
2. The use of monoclonal antibodies, generated against human mammary tumor cells, to identify antigens associated with, or specific for, human mammary adenocarcinoma cells.
3. The generation of human monoclonal antibodies (by the fusion of murine nonproducer [NS-1] myeloma cells with human lymph node cells obtained from modified radical mastectomy patients) against human mammary tumor associated or mammary tumor specific antigenic determinants.

BACKGROUND AND RATIONALE: Tumor specific antigens are molecules expressed uniquely on cells of a tumor. Their presence or absence on cells of a given tumor, and their immunogenicity in a particular host determines to what extent, if at all, the host's immune response will be involved in tumor host interrelationships and eventually tumor control. Developing highly specific reagents for these antigens is probably the most promising single approach for reliable and highly sensitive tumor detection, diagnosis, and possibly for specific tumor therapy or immunoprevention. Recently, the development of techniques to generate practically unlimited amounts of monoclonal antibodies directed against a single antigenic determinant has brought this class of biological reagents to the highest imaginable level of specificity, and at the same time has eliminated many ambiguities due to cross reactions. We are at present using the by now classical cell fusion hybridoma technique to generate monoclonal antibodies to a variety of experimental murine mammary tumors as well as human breast cancer. By testing each monoclonal antibody in successive screening assays for reactivity with cell surface antigens, those monoclonals specific for putative tumor associated or tumor specific antigens can be singled out for further study.

MAJOR FINDINGS:

An extremely important component of any study employing monoclonal antibodies to identify unique cell surface antigenic determinants is the development of rapid, sensitive, and specific immunoassays for the detection of a hybridoma clone synthesizing the desired monoclonal antibody; we have developed several assays to this end. These include: (a) solid phase radioimmunoassays employing either virus or purified cell membrane fractions fixed to microtiter wells as a primary screen, (b) solid phase radioimmunoassays employing live monolayer cells growing in monolayer in microtiter plates as a further screen for specificity, and (c) immunoperoxidase assays using fixed tissue sections as a final screen

to define staining of tumor cells versus nontumor cells, both obtained from the same individual (to reduce HLA ambiguities, etc.), and both prepared in an identical manner. The assays developed for screening monoclonal antibodies utilize extremely low (ng) levels of virus or partially purified cell membranes (ug levels). The assays can detect approximately 0.1 ng of specific immunoglobulins. For the detection of mouse monoclonal antibodies ^{125}I-labeled F(ab')$_2$ fragments of rabbit anti mouse F(ab')$_2$ fragments of IgG or rabbit anti mouse F(ab')$_2$ fragments (followed by the addition of ^{125}I-labeled Staphylococcus aureus Protein A [SpA]) are used. For the detection of rat monoclonal antibodies, rabbit anti rat F(ab')$_2$ fragments or rabbit anti rat IgG (heavy and light chains) are bound followed by ^{125}I-SpA. In the live cell assay, goat anti mouse F(ab')$_2$ is sequentially followed by pig anti goat serum, rabbit anti pig, and finally ^{125}I-SpA. Degree of binding is assayed by autoradiography or by counting each individual well.

The cytologic localization of a variety of antigens has previously been studied by the use of enzyme-labeled antibodies. The most widely used reagent for such studies has been an enzyme-antibody conjugate involving horseradish peroxidase. Peroxidase-conjugated antibodies have been used in either a direct or an indirect test to examine the distribution of antigens within or on a cell or tissue. In the direct method, the antibodies specific for the antigen are conjugated with peroxidase. In most cases, the indirect method in which an enzyme labeled anti-antibody is used, allows more flexibility and sensitivity for the detection of a variety of different primary antibodies. Since monoclonal antibodies have such restricted ranges of reactivity, the quantity of the specific antigenic determinants on the tumor cell surface detectable by them may be much lower in comparison to those recognized by more conventional multivalent antisera. In order to maximize the sensitivity of the immunoperoxidase method for the detection of low antigen levels by monoclonal antibody, comparisons were made between three different indirect methods of the immunoperoxidase technique: (a) the use of enzyme conjugated anti-gamma globulin to bind the primary antibody; (b) the sequential use of an unlabeled anti-gamma globulin followed by a second anti-gamma globulin conjugated with enzyme; (c) the use of two anti-gamma globulins followed by the peroxidase anti peroxidase reagent (PAP). Serial dilutions of various mouse monoclonal antibodies to MMTV glycoprotein (gp36) were incubated with histologic sections of a variety of mouse mammary tumors. The gp36 molecule was employed as a prototype tumor associated antigen since it is found only on mammary cell surfaces and not normal stroma. The endpoint dilution and intensity of staining of the primary mouse monoclonal antibody was then determined by each of the three detection methods. The third method described above greatly increased both the sensitivity and intensity of staining by primary mouse antibody; the specificity of the reaction was retained. Thus, this method involving the sequential stacking of immunospecific reagents is now being employed.

All of the above assays are now being employed toward the detection of virion or nonvirion mammary tumor associated or specific antigenic determinants. Several monoclonal antibodies recently generated have now been characterized; they react specifically with partially purified membranes from C3H mammary tumor cells and in immunoperoxidase assays with cells of spontaneous mammary tumors of several strains of M. musculus, as well as cells of mammary tumors of M. cervicolor and M. cookii.

Experiments are now in progress to extend preliminary results using several monoclonal antibodies that react with C3H and GR mammary tumor cells and not normal murine cells, and of antibodies that react with MCF-7 human mammary tumor cells and not normal human cells. The various assays described above are now being employed to further characterize the distribution of the antigenic determinants recognized by these antibodies.

Human Monoclonal Antibodies.

Studies are currently in progress to generate human monoclonal antibodies to putative human mammary tumor associated- or tumor-specific antigens. The rationale for this study is that B-lymphocytes, obtained from axillary nodes removed at the time of modified radical mastectomy, may be primed for antibody synthesis against putative mammary tumor specific antigenic determinants. We have developed specific and sensitive competition radioimmunoassays for human immunoglobulins. Hybridomas secreting human immunoglobulins are detected by their ability to compete for the binding of ^{125}I-labeled human immunoglobulin (IgG, IgM, or IgA) to rabbit anti human Ig (IgG, IgM, or IgA for specific assays, respectively) bound to polyacrylamide microspheres. Employing these assays we can detect as little as five ng of human immunoglobulin. As much as 10^4 ng of murine immunoglobulin does not compete in these assays.

Segments of axillary lymph nodes (otherwise discarded by pathologists following removal of segments for pathological diagnosis) are being obtained within hours of surgery and fused with murine NS-1 nonproducer myeloma cells. Employing standard hybridoma cultivation procedures, human-mouse hybrids have been established, cloned, and cultivated for a total of up to 130 days (experiments still in progress). Many of the isolated clones have been shown to be producing either human IgG or IgM for as long as up to 130 days in culture. The average yield of human immunoglobulin is 5-10 ug/ml. The human-mouse hybrids replicate at approximately the same rate as mouse-mouse or mouse-rat hybrids. Studies are now in progress, employing the radioimmunoassays and immunoperoxidase techniques described above, to analyze the reactivity of these human monoclonal antibodies with mammary tumor cells in paraffin sections of human mammary tumors. Some of the human immunoglobulins synthesized showed reactivity by the immunoperoxidase technique with human mammary tumors (including the tumor of the patient from which the segments of nodes were obtained). These same antibodies did not react with normal mammary epithelium found in the same breast; the specificity of this reactivity is currently under investigation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROPOSED COURSE: The development of the hybridoma technique for the generation of monoclonal antibodies to unique and specific cellular antigenic determinants has, and will continue to have, wide application in the detection and characterization of tumor associated and unique tumor specific antigens. Our studies on the generation of monoclonal antibodies to detect viral and nonviral tumor-associated, or tumor-specific antigens associated with murine mammary tumors will, if successful, undoubtedly aid in elucidating the host's immune response and its role in spontaneous mammary neoplasia. They will also be employed in therapy trials to determine the effect of monoclonal antibodies on the growth of both primary and metastatic mammary tumor cells. Our studies on the generation of monoclonal antibodies to putative human mammary tumor cell associated or specific antigens, if successful, may have direct application to the diagnosis and prognosis of primary or metastatic mammary cancer. Our studies with human monoclonal antibodies are directed toward the identification of tumor antigens that may be specific for each individual tumor. The potential for use of these human monoclonal antibodies in therapy also merits consideration.

PUBLICATIONS:

Teramoto, Y. A. and Schlom, J.: Radioimmunoassays for the 36,000 dalton glycoprotein of murine mammary tumor viruses demonstrate type, group and interspecies determinants. J. Virol. 31:334-340, 1979.

Schlom, J.: Type-B and type-D retroviruses. In Stephenson, J. (Ed.): Molecular Biology and RNA Tumor Viruses, Chapter 11. New York, Academic Press, Inc., 1980, pp. 447-484.

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Drohan, W., Cardiff, R. D., Lund, J. K., and Schlom, J.: Correlation between the detection of specific mouse mammary tumor proviral sequences and the presence of pulmonary metastases in mice bearing spontaneous mammary tumors. Cancer Res. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05136-01-LVC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Detection of Mammary Tumor Virus Interspecies Antigenic Determinants

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. Teramoto	Senior Staff Fellow	TVDS LVC NCI
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COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Tumor Virus Detection Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of these studies is to utilize viruses isolated from milk and spontaneous mammary tumors of Mus species other than that of M. musculus (the laboratory mouse) to: (i) examine the relationship between spontaneous mammary cancer and type-B retroviruses in these species; and (ii) develop immunoassays that detect conserved interspecies determinants of type-B retroviruses to search for related viral gene products in more distant species. Major accomplishments during this past year include: (a) the isolation and characterization of the first type-B retroviruses to be isolated from species other than M. musculus, and (b) the development of the first interspecies RIAs for mammary tumor viruses (MTVs). These assays, for the major envelope proteins and the major internal protein of MTVs have determined the distribution of these and related gene products in other species. These assays employ both conventionally raised heterologous sera and monoclonal antibodies generated to conserved antigenic determinants with interspecies reactivities. These studies have expanded the spectrum within which MTV etiologically related gene products can be analyzed.

PHS-6040
(Rev. 10-76)

Project Description

OBJECTIVES: To utilize viruses isolated from milk and spontaneous mammary tumors of Mus species other than that of M. musculus (the laboratory mouse) to (i) examine the relationship between spontaneous mammary cancer and type-B retroviruses in these species, and (ii) develop immunoassays that detect conserved interspecies determinants of type-B retroviruses to search for related viral gene products in more distant species. Specific aims include:

(a) Characterization of type-B retroviruses isolated from M. cervicolor, M. cookii, and M. caroli and determination of correlations between their distribution and mammary cancer in their respective species.

(b) Development of interspecies competition RIAs for the gp52 and gp36 major envelope proteins and p28 major internal proteins of mammary tumor viruses (MTVs) and employing these assays to determine the distribution of these and related gene products in other species. These assays will employ both conventionally raised heterologous sera and monoclonal antibodies generated to conserved antigenic determinants with interspecies reactivities.

BACKGROUND AND RATIONALE: Mouse mammary tumor viruses (MMTVs) have been isolated from numerous strains of the common laboratory mouse M. musculus. They possess distinct morphological (termed type-B), immunological, and biochemical properties that distinguish them from both type-C and type-D retroviruses. MMTVs isolated from different strains of M. musculus can be distinguished from one another by their immunological properties and RNA genomes. All MMTVs thus far isolated, however, possess group-specific antigenic determinants that reside on the 28,000-dalton major internal protein (p28) and the 52,000-dalton and 36,000-dalton external glycoproteins (gp52 and gp36) of the virion. Whereas MMTV studies have proved valuable in elucidating factors involved in the etiology of mammary neoplasia, a major problem has been the absence of viral isolates related to MMTV from species other than M. musculus.

M. cervicolor, M. cookii, and M. caroli are Asian rodents believed to have diverged from M. musculus several million years ago. Recently, two type-C retroviruses, designated CERV-CI and CERV-CII, and a virus designated M432 have been isolated from M. cervicolor. The M432 virions share some properties in common with MMTV, but can be clearly distinguished from type-B, C, and D retroviruses by morphological, biochemical, and immunological criteria. The studies reported here present an excellent opportunity to conduct etiological studies of mammary cancer in a "feral" (i.e., recently trapped) outbred population of rodents of a species other than M. musculus.

MAJOR FINDINGS: A virus similar by many parameters to the murine mammary tumor viruses (MMTVs) of the laboratory mouse M. musculus, has been identified in the milk of M. cervicolor popaeus mice. This virus is morphologically indistinguishable from the type-B MMTV and was thus termed

M. cervicolor mammary tumor virus (MC-MTV). MC-MTV has a density of 1.16 g/ml in sucrose and a virion-associated DNA polymerase with a divalent cation preference for Mg^{2+} over Mn^{2+}. Radioimmunoassays clearly differentiate MC-MTV from the other viruses previously identified from M. cervicolor, i.e., M432, CERV-CI, CERV-CII. Particles similar to MC-MTV were also observed in numerous spontaneous M. cervicolor popaeus mammary tumors.

To determine if antigenic determinants were shared by MC-MTV and MMTV, competitive radioimmunoassays for MMTV gp52, gp36, and p28 were employed. In a "group-specific" assay (antisera prepared against MMTV(C3H) gp52 and ^{125}I-labeled MMTV(RIII) gp52), all MMTV isolates examined from various inbred strains of M. musculus competed completely and with the same slope. In contrast, purified MC-MTV competed only partially and with a reduced slope. Similarly, in a "group-specific" RIA for the gp36 of MMTVs, all MMTVs competed completely whereas MC-MTV yielded a competition curve with a reduced slope. No reactivity was observed in the above immunoassays with M432 virus, CERV-CI and CERV-CII viruses, murine type-C viruses, and C57BL milk (a strain of mouse whose milk is devoid of MMTV particles).

We have also recently developed a "group-specific" RIA for the p28 major internal protein of MMTVs. In this immunoassay, the addition of increasing inputs of density gradient-purified, disrupted MC-MTV, resulted in only partial blocking with a reduced slope. A greater degree of competition with a steeper slope was consistently observed, however, when MC-MTV was used as competitor in the p28 RIA than in the gp52 RIA. To obtain a more accurate comparison of slopes using different competitors, we plotted the linear portion of the displacement curves in logit form, using MMTV from C3H, GR, and RIII mice and MC-MTV as competitors. When the MMTV p28 group-specific RIA data were plotted in this manner, MC-MTV was easily distinguished from MMTV(C3H), MMTV(GR), and MMTV(RIII). These studies have thus identified the first virus from another species that is immunologically related to the carcinoma inducing viruses of M. musculus.

The cross reactivity between antigens of MC-MTV and MMTVs suggested the development of interspecies immunoassays for mammary tumor viruses. The development of such assays, as in the case of type-C and type-D retroviruses should be successful in detecting related antigenic determinants and/or viruses in still other species. The antigenic determinants shared by MMTV gp52 and MC-MTV were examined by two different gp52 RIAs. In one assay, antiserum to MC-MTV was used to bind ^{125}I-labeled MMTV gp52. In the reciprocal gp52 RIA, antiserum to MMTV gp52 was used to bind ^{125}I-labeled MC-MTV intact virions. In the former gp52 RIA, less than 50 pg of purified MMTV gp52 was required to show competition. As described above, MC-MTV competes incompletely in the group-specific MMTV gp52 RIA. The complete competition and the similarity in slopes of the MMTV and MC-MTV competition curves, in the interspecies gp52 RIA, however, strongly suggests that similar, if not identical antigenic determinants are being measured. The interspecies gp52 RIA also

demonstrated complete inhibition by extracts of MMTV-infected feline CrFK cells, but not by extracts of uninfected CrFK cells. The specificity of this interspecies gp52 RIA was further demonstratd by the inability of purified MMTV gp36 and MMTV p28 to compete. Murine type-C viruses, M432, CERV-CI virus and CERV-CII virus, bovine leukemia virus and guinea pig retrovirus also did not compete. Milk and lactating mammary gland extracts of C57BL mice and fetal calf serum proteins also did not compete. The interspecies gp52 RIA was then used to determine if milks from different murine species contain cross reactive antigenic determinants. Milks were obtained from the outbred Asian feral rodents, M. cookii and M. caroli. These animals, along with M. cervicolor mice used in these studies were recently trapped in various provinces in Thailand.

A great variation in the amount of immunoreactive protein was observed with M. cookii, M. caroli, and M. cervicolor milks occasionally demonstrating as much as four to 40 ug of immunoreactive virus per ml of milk when scored in the interspecies RIA. These values correspond to approximately 10^{10} to 10^{11} particles per ml. Since these are outbred mice that have been recently trapped in the wild, one would expect the variation in titers observed.

An RIA was also developed to detect the interspecies antigenic determinants shared by MMTV gp36 and MC-MTV. In this assay, antiserum against MC-MTV was used to bind purified ^{125}I-MMTV gp36; as little as 0.15 ng of input protein was required for 20% competition. Feline-grown MMTVs competed completely and with identical slopes, providing further evidence that the assay is measuring viral-coded determinants. Purified MC-MTV and MCo-MTV (obtained from M. cookii milk) also completely inhibited the binding in this assay. Extracts of spontaneous mammary tumors from M. cervicolor and M. cookii also showed significant competition in the interspecies gp36 RIA. In contrast, extracts of livers from M. cervicolor, M. cookii, and M. musculus did not block at protein input concentrations as high as 10^5 ng.

We have also recently developed an interspecies RIA which detects antigenic determinants shared by the major core protein of M. musculus and MMTVs isolated from M. cervicolor, and M. cookii. Protein extracts of spontaneous mammary tumors from M. cervicolor, M. cookii, and M. musculus were also used as competitors in the interspecies MTV p28 RIA; tumors from each species exhibited a range of titers. This is most probably contributed to by the variation in the percent of actual tumor cells versus stroma and infiltrate observed in any spontaneous mammary carcinoma. Further studies are in progress to determine if factors such as histologic type of tumor affect the presence or the amount of MTV antigen in individual tumor samples. No detectable interspecies MTV p28 reactivity was observed in protein extracts of livers or other apparently normal tisues examined from M. cervicolor, M. cookii, and M. musculus.
We have recently developed monoclonal antibodies specific for the interspecies antigenic determinants shared by the MMTVs of M. musculus and

MC-MTV or MCo-MTV. Some of these shared antigenic determinants appear to be highly conserved among a variety of as yet undetected MTVs from other mammalian species. Monoclonal antibodies have been generated by immunizing C57BL/6 mice with either detergent-disrupted MC-MTV or MCo-MTV. After fusion of the spleens from these mice with the NS-1 non-producing mouse myeloma cell line, hybridoma cell lines were initially selected for the preferential production of antibody directed against MMTV and not MuLV or AMV. Thus, antibodies directed against MMTV proteins were, by definition, detecting interspecies determinants since they were generated against MTVs isolated from species distinct from the species of origin of MMTVs. These cell lines have been cloned twice by limiting dilution and the antibodies they produce have been characterized. At least 15 hybridoma cell lines derived in this manner are producing immunoglobulins of the IgG_1 class, that react specifically and with high titer against purified MMTVs and MC-MTV. These data were obtained using a solid phase immunoassay recently developed. These monoclonal antibodies were also positive against detergent-disrupted MMTV virions obtained from culture fluids of feline cells infected with MMTV, indicating that the reactivity detected against wild-type MMTV was not due to anti-murine cellular antibodies. The hybridoma cell lines are consistently negative when detergent-disrupted type-C and type-D retroviruses are used as test antigens in these immunoassays. Studies are now in progress to develop interspecies immunoassays employing these monoclonal antibodies. These antibodies are potentially valuable reagents in the detection of MTV-related translation products in mammary carcinoma tissues of a variety of mammalian species.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROPOSED COURSE: A major problem in MMTV research is the lack of comparative virology. The studies involved in this project have defined, for the first time, that viruses immunologically and morphologically related to the known carcinoma inducing MMTVs, exist in species other than M. musculus. The existence of these viruses in recently trapped feral mice from Thailand demonstrate that MMTVs are not laboratory artifacts of inbred M. musculus strains. The colonies of M. cervicolor, M. caroli, and M. cookii in existence also present an excellent alternative model for studies concerning a possible viral involvement in the etiology of spontaneous mammary cancer. The interspecies RIAs developed for the gp52, gp36, and p28 of MTVs now make possible the study of the expression of MTV-related translational products in these colonies, as well as in the detection of related determinants in spontaneous mammary tumors of other species. The existence of MC-MTV and MCo-MTV also now greatly simplifies the generation of monoclonal antibodies to MTV interspecies determinants, since animals can be immunized to antigens of either of these viruses, and the resulting hybridoma clones can be tested for reactivity to purified MMTV proteins. These monoclonal antibodies can now be employed in RIAs to search for specific, highly conserved, MTV-related translational products in more distant species.

PUBLICATIONS:

Teramoto, Y. A. and Schlom, J.: Radioimmunoassays for the 36,000 dalton glycoprotein of murine mammary tumor viruses demonstrate type, group and interspecies determinants. J. Virol. 31:334-340, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05138-01-LVC

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Identification of Genes Associated with Mammary Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. Drohan	Senior Staff Fellow	TVDS LVC NCI
	J. Schlom	Section Head	TVDS LVC NCI
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COOPERATING UNITS (if any)

None

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SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of these studies is to identify, characterize and isolate endogenous genes of mice that correlate with the Mendelian segregation of susceptibility to mammary cancer. During this past year, we have shown that the segregation of mammary tumorigenesis in crosses of GR mice resembles that observed in certain high risk breast cancer families, i.e.,[C57BL x (C57BL x GR)F1] backcross (BC1) mice have a 50% incidence in breast cancer. We have defined the mammary tumor virus (MMTV) genetic information in C57BL, GR, F1, and BC1 mice by both liquid hybridization and restriction endonuclease analyses. This has led to the identification of those endogenous genetic elements of GR mice that give a 100% correlation with the segregation of susceptibility to mammary tumorigenesis. Restriction endonuclease analysis of DNA from blood samples of intact young adult female mice have revealed which siblings of a given litter will eventually develop mammary cancer within one year. This system, therefore, has also provided an excellent in-vivo model to distinguish among tumor initiations, cocarcinogens, and tumor promotion.

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(Rev. 10-76)

Project Description

OBJECTIVES: The purpose of these studies is to identify, characterize, and isolate endogenous genes of GR mice that correlate with the Mendelian segregation of susceptibility to mammary cancer. Specific aims are as follows:

(a) Define the MMTV genetic information in C57BL, GR, F_1, and BC_1 mice by liquid hybridization and restriction enzyme analyses.

(b) Employ liquid hybridization and restriction enzyme analyses to define and isolate those endogenous genes segregating with mammary tumorigenesis.

(c) Molecular cloning of the genetic elements defined above for the preparation of probes. These will be employed to determine if the transcription of these sequences correlates with malignancy of the mammary gland.

BACKGROUND AND RATIONALE: Several studies have demonstrated that members of certain families exhibit a susceptibility to mammary cancer that follows a single Mendelian gene segregation pattern. An analogous situation exists in certain offspring of the GR mouse strain. It has been shown that when GR mice (100% mammary tumor incidence in females) are crossed with C57BL mice (0% incidence), the resulting (C57BL x GR)F_1 mice have 100% mammary tumors. When the F_1 mice are backcrossed to C57BL, the resulting female BC_1 mice, i.e., [C57BL x (C57BL x GR)], have 50% mammary tumor incidence before one year of age. It was therefore postulated that early mammary tumorigenesis in this strain is segregating as a single Mendelian unit; the inference was that this was the result of an MMTV gene. Several studies have demonstrated that both GR and C57BL mice contain MMTV proviral DNA. Our studies with tumor associated sequence (TAS) probe and restriction analysis, however, demonstrated that C57BL DNA is devoid of these particular sequences, whereas the GR strain possesses them as endogenous genetic elements. The possibility was thus explored that the acquisition of these genes may correlate with the presence of susceptibility to early occurring mammary cancer.

MAJOR FINDINGS:

Since tumor-associated sequences (TAS) have been shown to be associated with early mammary tumorigenesis in strains such as C3H and RIII, experiments were undertaken to determine (1) if TAS proviral sequences are segregating as a single Mendelian unit in [C57BL x (C57BL x GR)F_1]BC_1 mice, and (2) if the presence of endogenous TAS in the DNA of normal tissue of a given BC_1 animal is associated with the occurrence of early mammary tumorigenesis. DNAs of the BC_1 mice positive and negative for TAS were also hybridized to radioactive MMTV cDNA or 60-70S RNA probes; these probes, however, were unable to distinguish the two BC_1 groups. This is not surprising, since both probes also could not clearly distinguish parental GR and C57BL mice. All of the DNAs of GR mice tested individually with radioactive TAS RNA probe were positive, and all

those of C57BL mice were negative; likewise, all of the (C57BL x GR)F_1 mice contained proviral sequences homologous to the TAS. The [C57BL x (C57BL x GR)F_1]BC_1 mice, however, segregated into two populations: seventeen out of the 33 mice tested contained the TAS sequences in normal cellular DNA, and sixteen did not contain the sequences. The presence of the TAS in DNA of 51.5% of the first backcross generation therefore indicates transmission as a single Mendelian unit.

C_0t analyses were performed using radioactive TAS probe, on DNAs of normal tissues of GR, (C57BL x GR)F_1, and TAS positive BC_1 mice; C_0t 1/2 values demonstrated approximately six copies of TAS in GR mice and three copies in F_1 and positive BC_1 mice. To further rule out the possibility that copy number effects were contributing to the segregation of TAS genes, DNA saturation curves were performed with the DNA from GR, C57BL, F_1, and BC_1 (pools of TAS positive and TAS negative) mice. Increasing the DNA input levels to as high as 1,200 ug for C57BL mice or TAS negative BC_1 mice, did not result in hybridization above background levels.

Spontaneous mammary tumor incidence at 42 weeks was approximately 95% in F_1, and 50% in BC_1 females. When 33 BC_1 mice were randomly sacrificed as weanlings (two mice per litter), 51.5% of mice (8 of 16 females and 9 of 17 males) contained proviral sequences homologous to TAS. To determine if there was indeed a correlation between the occurrence of an early mammary tumor and the presence of TAS provirus, the first fourteen females to develop mammary tumors were sacrificed and DNAs from normal tissues (both liver and kidney) were hybridized to radioactive TAS probe. All tissues were positive for TAS. The probability of randomly associating sequences (i.e., TAS) being present in the DNA of 100% of the first fourteen mice tested is 3×10^{-5}. Mammary tumors of these mice were also all positive for MMTV antigens by immunoperoxidase. These studies thus demonstrate (1) segregation of specific MMTV genes (homologous to TAS) as a single Mendelian unit in GR and C57BL crosses, and (2) an association between the acquisition of specific proviral sequences, via normal processes of Mendelian segregation of genes, and the occurrence of early mammary tumors in individual animals. DNA extracted from kidney biopsy or extracted from blood samples of apparently normal weanlings or young adult BC_1 mice, can be used to determine which siblings of a given litter will eventually develop mammary cancer.

Restriction endonuclease studies have demonstrated that Pst-1 cuts MMTV genomic sequences internally. We have employed this enzyme to examine GR, C57BL, F_1 mice and BC_1 mice. There is a 100% correlation between the existence of Pst-1 bands at 2.5 and 0.6 megadaltons and TAS in DNAs of BC_1 mice.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROPOSED COURSE: These studies are aimed at the identification of endogenous genetic elements, that are responsible for the Mendelian segregation of susceptibility to mammary

cancer. It must be emphasized that only the physical isolation of these genetic elements, and their demonstrated ability to induce mammary cell transformation by transfection studies, will prove the existence of a "mam" gene. The system as it is now developed, however, provides an excellent in-vivo model to differentiate tumor initiation from promotion. DNA of blood samples obtained from weanling or young adult BC_1 mice will now reveal (by restriction analyses) which siblings of various litters will develop mammary cancer before the age of one year. This provides an excellent opportunity to determine which substances will initiate additional tumor formation (in mice negative for TAS or certain restriction fragments) versus those substances which will promote an earlier occurrence of mammary tumors in those animals positive for these specific genomic elements. Molecularly cloned MMTV fragments can now also be employed in this model as probes to determine differential degrees of transcription of specific sequences in the two BC_1 populations. Finally, these studies begin to shed some insight into the molecular events that manifest themselves in the Mendelian segregation of susceptibility to mammary cancer.

PUBLICATIONS:

Drohan, W., and Schlom, J.: Differential distribution of mouse mammary tumor virus-related sequences in the DNA of rats. J. Natl. Cancer Inst. 62:1279-1286, 1979.

Drohan, W., and Schlom, J.: Diversity of mammary tumor viral genes within the genus Mus, the species Mus musculus and the strain C3H. J. Virol. 31:53-62, 1979.

Schlom, J.: Type-B and type-D retroviruses. In Stephenson, J. (Ed.): Molecular Biology and RNA Tumor Viruses. Chapter 11, New York, Academic Press, Inc., pp. 447-484, 1980.

Schlom, J., Colcher, D., Drohan, W., Horan Hand, P., Howard, D., and Teramoto, Y. A.: Systematics of murine mammary tumor viruses. In Brennan, M., Rich, M. A., and McGrath, C. M. (Eds.): Advances in Breast Cancer Research. New York, Academic Press, Inc. (In Press).

Schlom, J., Drohan, W., Teramoto, Y. A., Young, J. M., and Horan Hand, P.: Diversity of mammary tumor viral genes and gene products in rodent species. In Cold Spring Harbor Conferences on Cell Proliferation - Viruses in Naturally Occurring Cancer, Vol. 7 (In Press).

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Drohan, W., Cardiff, R. D., Lund, J. K., and Schlom, J.: The detection of specific mouse mammary tumor proviral sequences correlates with the presence of pulmonary metastases in mice bearing spontaneous mammary tumors. Cancer Res. (In Press).

Drohan, W., Young, J., and Schlom, J.: Correlation between the development of murine mammary cancer and the segregation of endogenous genes. In Fields, B., Jaenisch, R., and Fox, C. F. (Eds.): Animal Virus Genetics, ICN-UCLA Symposia on Molecular and Cellular Biology. New York, Academic Press, Inc., Vol. XVIII (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1-CP-05140-01-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Determinants of Tumor Cell Phenotype

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N.H. Colburn	Expert	LVC NCI
	D.D. Dion	Staff Fellow	LEP NCI
	L. Srinivas	Visiting Fellow	LVC NCI

COOPERATING UNITS (if any)
Dr. R. Tucker, Johns Hopkins Medical School; Dr. Henry Hennings, LEP, DCCP, NCI

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.5	0.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this research is to identify in vitro correlates of tumorigenicity which characterize the phenotype of tumor cells, to use these endpoint assays in transformation studies, and to ascertain their role maintaining the tumor cell phenotype. Recently published results indicate that anchorage independent growth shows a consistent correlation with tumorigenicity of mouse epidermal cell lines. These epidermal cell lines are being characterized with respect to presence of epidermal specific cell surface antigen, quantitative measures of tumorigenicity, levels of plasminogen activator activity, serum density and adhesion dependence of growth, cell surface glycoconjugate profiles, and growth factor and promoter receptor levels.

PHS-6040
(Rev. 10-76)

. Project Description

Objectives:

The objective of this research is to identify in vitro correlates of tumorigenicity which characterize the phenotype of tumor cells, to use these as endpoint assays in transformation studies, and to ascertain their role in maintaining the tumor cell phenotype. Specific aims:

1. To characterize cell lines obtained after exposure of primary mouse epidermal cell cultures to carcinogens with respect to tumorigenicity, growth in 0.33% agar, growth in low calcium medium, levels of proteases, particularly plasmenogen activator, changes in specific cell surface glycoproteins and gangliosides, and responsiveness to external regulators or cell interactions.

2. To ascertain how consistently each of these properties correlates with tumorigenicity (assayed in nude or syngeneic mice) and to utilize the most reliable ones for transformation end point assays.

3. To investigate the role of some of the parameters listed in #1 specifying tumor cell phenotype.

Methods Employed:

Measurement of parameters listed under specific aim #1.

Major Findings:

The Model System. The newborn mouse primary epidermal cell culture system of Yuspa and coworkers has been used for carcinogenesis in vitro studies (Colburn et al., Cancer Research, 1978, Raven Press, 1978). Cultures were exposed to alkylating carcinogens or to polycyclic aromatic hydrocarbons under various conditions and long term cell lines were established. Exposure of primary cultures at the time of plating to sufficiently high concentrations of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or 7, 12 dimethylbenz (a) anthracene (DMBA) led to a high proportion (about 75%) of dishes which gave rise to tumorigenic cell lines. No tumorigenic cell lines arose from solvent controls. Cultures have been obtained from both Balb/C which are relatively insensitive and Sencar mice which have been bred for sensitivity to a DMBA Phorbolester (TPA) scheme of 2 stage carcinogenesis. (Raven Press, 1978). Both Sencar and Balb/C cell lines are available for studies of determinants of carcinogen or promoter sensitivity.

Evidence for the epidermal origin of the cell lines has been obtained from studies of epidermal specific cell surface antigen. (Colburn, Academic Press, 1979). All lines assayed showed moderate to high levels of epidermal antigens with no clear distinction between tumor and nontumor cell lines.

In Vitro Correlates of Tumorigenicity. To date colony formation in 0.33% has been the most reliable correlate of tumorigenicity for mouse epidermal cell lines. (Cancer Research, 1978). Tumorigenicity assays have been carried out on some thirty cell lines by injection into syngeneic (Balb/C) or nude mice, with the result that anchorage independent lines consistently formed tumors while the anchorage dependent lines did not. Tumor cell colony yield in agar was 10 to 80% depending on cell line and serum concentration. Recently, a highly tumorigenic cell line has been identified which gives only a very low yield of larger than average agar colonies. This together with other published data suggests that capacity to grow independently of anchorage is not a cause of tumorigenicity. Nevertheless the possibility needs to be investigated that whatever determines tumorigenicity is an event which is coordinately regulated with anchorage independence most of the time.

Recent work suggests a trend toward correlation of tumorigenicity with high fibrinolytic activity. (Laug, Benedict, and Colburn, in preparation). Other proteases are also of interest. Resistance to low calcium is another parameter being measured. Preliminary results (Hennings and Colburn) indicate that resistance to low calcium concentration, a property which characterized a variety of fibroblastic transformants, may not be a consistent characteristic of tumorigenic epidermal lines. Work in progress deals with characterization of cell surface glycoproteins (Dion and Colburn, AACR, 1980) and glycolipds (Leela and Colburn) associated with tumor cell phenotype.

Determinants of Neoplastic Phenotype: We plan to begin work in this area during the next year.

Significance to Biomedical Research and the Program of the Institute:

Although tumorigenicity may be determined not at the cellular but at the tissue or organ level, the possibility of cellular determinants is certainly worth investigating. Furthermore, reconstituted culture systems can provide insight into the roles of cell and tissue interactions. Elucidation of cellular or molecular determinants of tumor cell phenotypes can lead to more rational approaches to reversal or prevention of malignancy.

Proposed Course:

The research to be pursued will focus on elucidating possible transforming gene products and on determining whether cell variants can be developed which permit dissociation of protease activity, cell configuration or glycoconjugate shifts from tumorigenicity.

Publications:

Colburn, N.H., Vorder Bruegge, W.F., Bates, J.R., Gary, R.H., Rossen, J.D., Kelsey, W.H., Shimada, T.: Correlation of anchorage-independent growth with tumorigenicity of chemically transformed mouse epidermal cells. Cancer Res. 38:624-634, 1978.

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Colburn, N.H.: The use of tumor promoter responsive epidermal cell lines to study preneoplastic progression. In Franks, L. M. and Wigley, C. B. (Eds.) Neoplastic Transformation in Differentiated Epithelial Cell Systems In Vitro. Academic Press, pp. 113-134, 1979.

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PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Preneoplastic Progression in Epidermal Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N.H. Colburn	Expert	LVC NCI
Other:	D.D. Dion	Staff Fellow	LEP NCI
	L. Srinivas	Visiting Fellow	LVC NCI

COOPERATING UNITS (if any) Dr. A. Jetten and Dr. L. De Lucca, LEP, DCCP, NCI; Dr. W. Laug and Dr. W. Benedict, Childrens Hospital, USC, Los Angeles; Dr. H. Herschman, UCLA, Los Angeles; Dr. B. Magun and Dr. T. Bowden, Univ. of Arizona, Tuscon, Arizona

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SECTION
Cell Biology Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The overall objective of this research is to elucidate the cellular and molecular basis of one or more stages which must occur during progression to malignancy in response to chemical carcinogens and tumor promoters. Rational approaches to prevention of one or more stages of progression is another long term goal. Recently published or in press results from our laboratory indicate that late stage tumor promotion by phorbol esters occurs by a process involving induction of a new phenotype (vs. selection of a preexisting variant). The mechanism appears not to require mitogenic stimulation or ornithine decarboxylase induction by the promoter. Promoter induced changes in cell surface glycoconjugate synthesis as well as chromosome and chromatid breaks and rearrangements occur and are being investigated for their role in promotion of tumor cell phenotype. Retinoids inhibit phorbol ester promotion in this system and are being used to investigate 1) required events in promotion and 2) cancer prevention.

PHS-6040
(Rev. 10-76)

Project Description

Objective:

The overall objective of this research is to elucidate the cellular and molecular basis of one or more stages which must occur during progression to malignancy in response to chemical carcinogens and tumor promoters. Rational approaches to prevention of one or more stages of progression is another long term goal. Specific aims:

1. To produce by carcinogen exposure a set of preneoplastic lines from mouse epidermal cells which will respond to tumor promoting phorbol esters by traversing one or more stages of progression.

2. To characterize the phenotype of cells which are the precursors or tumor promoter target cells for one or more stages of preneoplastic progression.

3. To elucidate the mechanism of one or more stages of promoter dependent prenoeplastic progression. Attention is focused on the role of inductive vs. selective processes, the role of mitogenic stimulation, and the roles of protease induction, cell surface alteration, cell interactions, transforming genes and their products and gene rearrangements.

4. To develop ways to prevent one or more promoter dependent stages by the use of retinoids, steroids or other antagonists.

Methods Employed:

Determination of anchorage independent growth (colony formation in 0.33% agar), mitogenic activity, glycoprotein and ganglioside synthesis responses to promoter and promotion inhibitors.

Major Findings:

The system: Recently we have reported on a mouse epidermal cell culture system which is being used to study late-stage preneoplastic progression in response to tumor promoters (Colburn et al., Nature, 1979). These promoter responsive cell lines undergo a concentration-dependent irreversible shift to anchorage independence in response to tumor promoting but not non-promoting phorbol esters. A series of cell lines has been established each from a soft agar colony induced by 12-O-tetradecanoyl phorbol-13-acetate (TPA) and found to retain the capacity to form colonies in agar in the absence of promoters. These anchorage independent transformants were tumorigenic (assayed in nude mice) while the precursors were not.

Induction vs Selection: Despite extensive clarification of the many activities of phorbol esters, the critical determinants of preneoplastic progression as mediated by tumor promoters remain to be elucidated. That tumor promotion occurs by a stagewise process is clear. Whether any of the

stages involves induction of new phenotypes by promoter versus selection of variants which preexist after carcinogen initiation has not previously been resolved. Recent studies (Colburn, accepted for publication, Science, 1980 and Colburn et al, Academic Press, 1979) show that the irreversible shift in anchorage independence by phorbol ester responsive mouse epidermal cell lines (JB-6) appears to occur not by a mechanism involving selection of preexisting transformants but by induction of a new phenotype, since a) the growth of transformants is not preferentially enhanced by phorbol ester and b) soft agar colonies result after exposure of single cells to phorbol ester. The magnitude of colony forming efficiency at one cell per well in response to TPA was as high as 37% in high serum, a level very similar to that found at 10^4 cells per dish, indicating that cells respond independently.

Advantages of the System: Two recent observations facilitate mechanism studies currently underway. The first is that the JB 6 cells show clonal heterogeneity for the anchorage independence response to TPA with a ten-fold difference between the highest and lowest responders (Colburn, Koehler, and Nelson, Teratogenesis, Carcinogenesis, and Mutagenesis Journal, in press, 1980). The clonal lines showed cross-sensitivity to several other non-phorbol promoters including mezerein, detergents, cigarette smoke condensate, and epidermal growth factor (EGF), which acts as a cocarcinogen if not a promoter in vivo. (TCM Journal, 1980). Thus the anchorage independence response appears to indicate promoting activity generally rather than a specific phorbol ester response.

Another observation which aids mechanism studies is that the TPA induced anchorage independence is inhibited by retinoids which inhibit tumor promotion in vivo. The activity of a series of retinoids for inhibiting anchorage independence induction parallels that for inhibiting tumor promotion (Colburn, Academic Press, 1979, TCM Journal, in press, 1980, and NY Acad. Sciences, 1980, in press).

Role of Mitogenic Stimulation and Ornithine Decarboxylase Induction: Recent studies on the mechanism of promoter dependent preneoplastic progression have focused on testing two hypotheses. The first is that motigenic stimulation is necessary for promotion. Although virtually all promoters are mitogens, the issue has been previously unresolved. Experiments in which mitogenic stimulation by TPA was prevented by maintaining cells in logarithmic growth during TPA exposure yielded no decrease in anchorage independence induction (Colburn and Ozanne, in preparation, and Colburn et al., N.Y. Acad. Sciences, in press, 1980). Nor did retinoic acid at concentrations which inhibit anchorage independence induction inhibit mitogenic stimulation by TPA at high density. Current efforts in selecting clonal lines which are resistant to the mitogenic action of TPA (Colburn and Wendel, in progress) are expected to further clarify the issue of the role of mitogenic stimulation in promotion. A related finding is that ornithine decarboxylase (ODC) induction appears not to be the target of TPA since TPA does not induce the enzyme in JB6 cells (Colburn, Lichti, Ben, and Yuspa, in preparation). However, ODC induction may

be involved in the process by which TPA induces anchorage independence, since retinoids inhibit the induction of ODC by medium change in these cells.

Role of Cell Surface Changes: A second hypothesis is that changes in specific cell surface molecules determine preneoplastic progression in response to tumor promoters. Although phorbol esters have been shown to produce changes in plasma membrane enzymes, glycolipids, fibronectin, and EGF receptors in several cell culture system, the systems did not permit relating the changes to the process of tumor promotion. The JB6 cells make possible studies on surface changes and preneoplastic progression in the same system. Recent studies have utilized the measurement of epidermal-specific cell surface antigens (Academic Press, 1979 and Colburn, Bosworth, Wenk, in preparation). Rabbit antiserum to primary mouse epidermal cells was prepared and made specific by in vivo absorption in mice. When compared for levels of epidermal cell surface antigen(s) in the mixed hemadsorption assay, a series of "normal", preneoplastic (TPA responsive for induction of anchorage independence) and neoplastic cell lines did not show a progressive loss. Nor did they show a change in epidermal antigen levels which correlated with the degree of TPA responsiveness. TPA treatment also produced no measurable changes in epidermal antigen. In other studies carried out with Todaro et al it has been found that cell surface receptors for EGF which are low in RNA virus transformed cells were also low in one out of four chemically transformed tumorigenic epidermal lines tested. A high TPA responsive JB6 clone showed moderately low levels while a low responsive clone showed high levels. If low-responsive and high responsive clones represent earlier and later stages of preneoplastic progression respectively, this suggests the possibility that one pathway of progression may involve progressive loss of EGF receptor. Additional studies currently underway deal with the role in promotion of changes in gangliosides (Leela and Colburn) and glycoproteins (Dion and Colburn) which 1) correlate with TPA responsiveness for anchorage independence induction and 2) are antagonizable by retinoids.

Role of Virus Genes and Gene Rearrangements: Electron microscopy studies carried out at the University of Michigan indicated that virus particle production occurred in some tumorigenic and some nontumorigenic cell lines. More recent assays of reverse transcriptase activity indicated that virus production occurred in several of our mouse epidermal cell lines but did not correlate with tumorigenicity or TPA responsiveness. Nor did TPA increase the reverse transcriptase levels in TPA responsive lines (Gallo and Colburn et al, unpublished).

Radman and others have postulated that tumor promoters (which are nonmutagenic) produce irreversible changes during promotion by causing gene rearrangements which occur after the promoter produces chromosome breaks (See the Review, Colburn, Raven Press, 1979). Radman, Little and others have presented evidence for enhancement of sister chromatid exchange (SCE) induction by promoters, and suggested that a mechanism analogous to this could lead to gene rearrangements. We have found that several SCE inducers are all inducers of anchorage independence in the TPA responsive JB6 cells. (Colburn, Koehler, and Bradley, in progress).

Significance to Biomedical Research and the Program of the Institute:
If one or more stages of preneoplastic progression can be recognized and if critical determinants of traversing that stage can elucidated this will make possible more specific and more rational schemes for cancer prevention.

Proposed Course:

Considerable evidence for the multistage nature of chemical carcinogenesis has been presented. The long latent period in human and aniaml systems together with studies using several systems (see Colburn, Raven Press, 1979) suggest that the process involves several stages which need to be traversed sequentially.

Considerable progress has been made in achieving specific aims #2, 3, 4. Work on (#3) the roles of mitogenic stimulation cell surface alterations, and gene rearrangements is continuing and constitutes active areas of research in the laboratory. In addition, new approaches to the development of promoter-responsive preneoplastic cells will begin.

Publications:

Colburn, N.H., VorderBruegge, W.F., Bates, J. and Yuspa, S.H.: Epidermal cell transformation in vitro. In Slaga, T. J., Sivak, A. and Boutwell, R. K. (Eds.): Mechanisms of Tumor Promotion and Carcinogenesis. Raven Press, pp. 257-271, 1978.

Colburn, N.H., Former, B.F., Nelson, K.A., Yuspa, S.H.: Tumor promoter induces anchorage independence irreversibly. Nature 281:589-591, 1979.

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Colburn, N.H., Ozanne, S., Ben, T., Lichti, U., Yuspa, S.: Inhibition promoter dependent preneoplastic progression by retinoids. Annals New York Academy of Sciences (in press), 1980.

Colburn, N.H.: Tumor promoter produces anchorage independence in mouse epidermal cells by an induction mechanism. Science (accepted for publication), 1980.

Dion, L.D., Colburn, N.H., De Luca, L.M.: Phorbol ester-induced anchorage independence may involve altered gycosylation of specific glycoproteins. Proc. Am. Assn Cancer Res. (Abstract), 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05142-01-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
To elucidate conserved sequence in genomes of RNA tumor viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Hatanaka	Expert	LVC NCI
Other:	Y. Tomita	Visiting Scientist	LVC NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A. The existence of a common conserved region of viral RNAs of various mammalian retroviruses was demonstrated for the first time by interviral molecular hybridization between fractionated viral complementary DNA and RNA.

B. The conservation and evolution of nucleotide sequences related to the 3'-end of retrovirus genomes in normal animal DNAs, including humans, have been found.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

A. To elucidate common sequences in genomes of retroviruses of distinct evolutionary origin via nucleic acid hybridization.
B. Analysis of the organization of common conserved region of retrovirus genomes in normal cell DNA.

Methods Employed:

Nucleic acid hybridization assays, including hydroxyapatite chromatography, Southern transfer and hybridization to blots. Restriction enzyme treatment to cleave cell DNA and gel electrophoresis.

Major Findings:

The genomes of avian RNA tumor viruses are an RNA of 10,000 nucleotides with polyadenylic acid (poly A) sequence of about 200 nucleotides at its 3'-terminus. Four genetic regions have been identified: gag, coding for internal structural proteins; POL, for RNA dependent DNA polymerase; eov, for coding of viral glycoproteins; and a gene for oncogenic transformation termed ooc. Recently, a fifth region has been found which is a common sequence of unknown function, located at the 3' terminus in RNAs of both avian leukosis and sarcoma viruses. Mammalian RNA tumor viruses are thought to contain a similar set of genetic regions. Although sequences common to the RNAs of Moloney sarcoma and leukemia viruses have been shown by heteroduplex analysis, there have been no reports of common sequences in the genomes of RNA tumor viruses in mammals. Since we have been investigating evolutional relationships of genes and gene produces of mammalian retroviruses, we attempted to find the existence of the common or conserved regions among mammalian retroviruses similar to those found for avian retroviruses.

A. The viral RNAs of various mammalian retroviruses contain highly conserved sequences close to their 3' ends. This was demonstrated by interviral molecular hybridization between fractionated viral complementary DNA (cDNA) and RNA. cDNA near the 3' end (cDNA3') from a rat virus (RPL strain) was fractionated by size and mixed with mouse virus RNA (Rauscher leukemia virus). No hybridization occurred with total cDNA (cDNAtotal), in agreement with previous results, but a cross-reacting sequence was found with the fractionated cDNA3'. The sequences between 50 to 400 nucleotides from the 3' terminus of heteropolymeric RNA were most hybridizable. The rat viral cDNA3' hybridized with mouse virus RNA more extensively than with RNA of remotely related retroviruses. The related viral sequence of the rodent viruses (mouse and rat) showed as much divergence in heteroduplex thermal denaturation profiles as did the unique sequence DNA of these two rodents. This suggests that over a period of time, rodent viruses have preserved a sequence with changes correlated to phylogenetic distance of hosts. The cross-reacting sequence of replication-competent retroviruses was conserved even in the

genome of the replication-defective sarcoma virus and was also located in these genomes near the 3' end of 30S RNA. A fraction of RD114 cDNA3', corresponding to the conserved region, cross-hybridized extensively with RNA of a baboon endogenous virus (M7). Fractions of similar size prepared from cDNA3' of MPMV, a primate type D virus, hybridized with M7 RNA to a lesser extent. Hybridization was not observed between Mason-Pfizer monkey virus and M7 if total cDNAs were incubated with viral RNAs. The degree of cross-reaction of the shared sequence appeared to be influenced by viral ancestral relatedness and host cell phylogenetic relationships. Thus, the strikingly high extent of cross-reaction at the conserved region between rodent viruses and simian sarcoma virus and between baboon virus and RD114 virus may reflect ancestral relatedness of the viruses.

B. The nucleotide sequences related to the 3'-terminal portion of retrovirus genomic RNA have been detected in the DNA of animals, including man. The DNA complementary to the 400-700 nucleotides from the 3'-terminus end of retrovirus RNA ($cDNA_{3'}$) that contains the enriched conserved region, was hybridized with DNA from a variety of animal cells. Under the conditions of annealing in 0.72 M NaCl at 67°C and hydroxyapatite chromatography at 55°C, 20 to 50% of the radioactivity of the $cDNA_{3'}$ prepared from two retroviruses, a murine virus (RLV) and a baboon virus (M7), annealed with normal cellular DNA of animals, including human tissue. The thermal denaturation profile revealed considerable mismatching between the duplex of the $cDNA_{3'}$ and human DNA. $cDNA_{3'}$ of retroviruses is most homologous to cellular DNA of the host species of origin, and is less homologous to cellular DNA of species that are distant in the phylogeny of the host species. The conservation and evolution of nucleotide sequences related to the 3'-end of retrovirus genomes in animal DNAs, including man, suggest that the sequences may have important functions.

Significance to Biomedical Research and the Program of the Institute:

A common conserved region of mammalian retrovirus RNA points to a common function during their life cycle. The presence of the "C" region in normal cellular DNA including humans suggests that the sequence is related to their oncogenic function.

Proposed Course:

1. The conserved region of retrovirus genome at the 3'-terminus will be further analyzed by molecular cloning from human library and subsequent subclonings in plasmid pBR322.

2. The probe of the conserved region after molecular cloning will be used for analysis of organization of the "C" region in human DNA and chromosomes.

Publications:

Kominami, R. and Hatanaka, M.: Conserved region of mammalian retrovirus RNA. J. Virol. 32:925-933, 1979.

Kominami, R., Tomita, Y., Connors, E.C. and Hatanaka, M.: The conserved sequence related to the 3'-terminal region of retrovirus RNAs in normal cellular DNAs. J. Virol. (in press), 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05144-01-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Polypeptide Growth Factor-Induced Alterations in Proliferating Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	U.I. Heine	Research Microbiologist	LVC NCI
Other:	J. Keski-Oja	Visiting Scientist	LVC NCI

COOPERATING UNITS (if any)
Dermatology Branch, NCI

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.5	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study was initiated to evaluate the mechanisms of action of polypeptide growth factors on epithelial cells of human and murine origin. The effects of epidermal growth factor (EGF) were found to be dose-dependent, dependent on the number of cell surface receptors, and they were reversible. EGF stimulates plasma membrane mobility and macropinocytosis and, concomitantly, enhances cell proliferation. The cell surface changes are sensitive indicators of the regulatory effects EGF exerts on epithelial cells. Our comparative studies indicate that epithelial cells of different origin respond differently to the growth factor. Although the receptor-rich human carcinoma cells of line A431 react immediately and intensively to EGF, the response of nontransformed murine cells (line MMC-E) results exclusively in a series of well defined morphologic events. Thus, these cells are especially well suited for the study of the effects of various growth factors and, furthermore, may serve as a model for the study of plasma membrane motility. Our results provide a basis for further studies in this research area.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To define the mechanism of action of epidermal growth factor (EGF) with respect to growth and morphology of cultured epithelial cells of human and murine origin.

Methods Employed:

Tissue culture techniques, phase and interference contrast light microscopy, transmission and scanning electron microscopy, tracer studies.

Major Findings:

It has been shown that cell surfaces contain specific receptor sites that interact with certain growth factors, especially mitogenic polypeptide hormones, a phenomenon that is followed by a series of events leading to DNA synthesis and cell division (Cohen, J. Biol. Chem. 237:1555, 1962). Moreover, induction of normal cells to express some of the properties of transformed cells has been achieved (De Larco and Todaro, PNAS (USA) 75:4001, 1978; Keski-Oja, De Larco, Rapp and Todaro, J. Cell Physiol. 103, 1980, in press). Both EGF and sarcoma growth factor (SGF) bind the same cell surface receptors and stimulate cell growth, but only SGF effects changes characteristic of transformation. Transformation of either epithelial or fibroblastic cells by murine or feline sarcoma viruses eliminates or greatly reduces the binding of EGF to cells (Todaro, De Larco and Cohen, Nature 264:26, 1976) suggesting that a product of the sarcoma virus might be functionally related to EGF and could compete for membrane receptor sites. The nontransformed mouse epithelial cell line MMC-E, as well as the human epidermoid carcinoma cell line A-431, apparently retain at least some potential for differentiation. MMC-E cells can form canaliculus-like structures in culture. They can also develop a transformed phenotype in response to SGF, and can be transformed both by type C viruses and by chemical carcinogens. After viral transformation these cells do not grow in organized cell islands and lack their distinctive morphology. Both cell lines are known to contain an extraordinarily high number of EGF receptors on their surface, i.e., A-431 has 2-3x106 (Fabricant, De Larco and Todaro, PNAS, USA, 74:565, 1977). We have taken advantage of this phenomenon in our studies concerning the mechanism of action of EGF on epithelial cells. We have concentrated in studying alterations in growth, shape, and topography of these cells due to the exposure to EGF since the regulation of these phenomena are currently a subject of interest in the study of transformation.

We have recently classified the murine MMCE-cell line as epithelial by the following criteria: morphological appearance, low levels of extracellular fibronectin, the demonstration of tight junctions and desmosomes at high resolution, and production of poorly differentiated carcinomas when inoculated in nude mice. The quantities of EGF that were sufficient in these cells to increase the incorporation of radioactive thymidine from the background level,

were about 100 picograms/ml, whereas nanogram quantities maximized the incorporation. Nanogram quantities were also necessary to obtain morphological changes at the cell surface. The topography of unstimulated MMC-E cells consisted of an extremely uniform pattern with an abundance of microvilli on the cell surface unrelated to the location of the cell in the islands of proliferating cells. Within seconds of exposure to the growth factor (100 ng/ml) ruffling of the plasma membrane and macropinocytosis occurred in numerous cells accompanied by the loss of microvilli. Ruffling subsided within 15 minutes and within one hour many cells again displayed numerous microvilli. Long term exposure to low doses of EGF (1-10 ng/ml) resulted in flattening of the cells, loss of microvilli and stimulation of multiplication as studied by thymidine uptake. The extreme flattening (or enlargement) of the treated cells was estimated by measuring the projected areas occupied by the cell colonies. A comparison of the number of cells per unit area showed that EGF-treated cells spread to about twice the area of control cells. Removal of the growth factor permitted the return to normal cell morphology. Non-transformed murine cells (MMC-E) and carcinoma-derived human cells (A-431) expressed morphological changes in different fashion. In nontransformed cells, the induced changes were very distinct. Our studies indicated that the growth factors stimulated membrane motility and macropinocytosis followed by enhanced cell division. The cell surface changes are sensitive indicators of the effects growth factors exert on cultured epithelial cells.

Significance to Biomedical Research and the Program of the Institute:

Our studies revealed that epithelial cells grown *in vitro* respond extremely rapidly to EGF and that this response has a distinct pattern of events in nontransformed murine cells. Thus, this cell system is extremely useful as a model to test the activity of other growth factors. Moreover, the system is now available to serve as a model for the study of plasma membrane motility, a phenomenon that remains as yet completely obscure. Thirdly, the results obtained thus far represent a basis for the projected investigation of the underlying events of the phenomena described here by studying, for example, the behavior of structural proteins, such as actin and tubulin, known to be decisive in cell surface changes in relation to alterations caused by the exposure to EGF and to mammalian tumor viruses. It is noteworthy, that similar changes have been observed in some avian cells infected with Rous sarcoma virus. These have been related to a virus coded phosphokinase similar to the actin binding kinase of mammalian sarcoma virus. Morphological observations of cytoskeletal changes may be of relevance in understanding the relationship of cytoskeletal organelles and their constituent proteins to the phenomenon of transformation by mammalian tumor viruses.

Proposed Course:

We are planning to investigate the underlying events of the observed phenomena by studying the behavior of structural proteins, such as actin and tubulin, known to be decisive in changes of cellular configuration. The results of our

investigations provide a basis for the study of the effects other growth factors, such as sarcoma growth factor and rat multiplication stimulating activity may exert on nontransformed and transformed cells. As soon as these factors will be available in sufficiently purified form their effects on controlled growth will be tested in our model system.

Publications:

Rapp, U.R., Keski-Oja, J. and Heine, U.I.: Establishment and characterization of the epithelial mouse embryo cell line MMC-E. Cancer Res. 39:4111-4118, 1979.

Keski-Oja, J., Heine, U.I., Rapp, U.R. and Wetzel, B.: Epidermal growth factor-induced alterations in proliferating mouse epithelial cells. Exp. Cell Res. (in press).

Heine, U.I., Keski-Oja., J. and Wetzel, B.: Ultrastructural alterations in cultured mouse and human epithelial cells induced by polypeptide growth factors. Proceedings 16th Ann. Mtg. AACR (1980), p. 42.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05147-01-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Aspects of the Association of Fibronectin with Cells and their Extracellular Matrices

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Keski-Oja	Visiting Fellow	LVC	NCI
OTHER:	C. J. Todaro	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The association of fibronectin with cultured cells and their matrices was studied by using models that included cultures of normal and malignant cells, isolated extracellular matrices of cultured human fibroblasts and purified protein components (actin, collagen, fibronectin). Specifically, (1) a 10,000 molecular weight peptide that causes the release of fibronectin from untransformed cells in culture was isolated, purified and characterized as well as a 66,000 molecular weight protein that was particularly sensitive to this fibronectin releasing peptide; (2) antibodies against this 66,000 molecular weight protein were produced; (3) the binding-site for actin in fibronectin molecules was isolated; (4) fibronectin-releasing 10,000 molecular weight peptides with respect to biological activities were identified. The production of these factors by different normal and malignant cells was studied.

Project Description

Objectives:

To study the association of fibronectin with cultured cells and their matrices by using models that include cultures of normal and malignant cells, isolated extracellular matrices of cultured human fibroblasts, and purified protein components (actin, collagen, fibronectin). Specifically, (1) to purify and characterize a 10,000 molecular weight peptide that causes the release of fibronectin from untransformed cells in culture as well a 66,000 molecular weight protein that is particularly sensitive to this fibronectin releasing peptide. These will hopefully allow the elucidation of the mechanism of fibronectin association to cultured cells; (2) to produce antibodies against this 66,000 molecular weight protein; (3) to isolate the binding-site for actin in fibronectin molecules; (4) to characterize further the fibronectin-releasing 10,000 molecular weight peptides with respect to biological activities. Study the production of these factors by different normal and malignant cells.

Methods Employed:

Immunological purification of fibronectin from cell extracts was accompanied by copurification of an actin-like protein. Some actin was also found in the cell-free fibronectin matrices of mouse fibroblasts. Cell surface-associated fibronectin is known to decrease in cells transformed by either RNA or DNA tumor viruses, by chemical carcinogens, or spontaneously. SV40 transformed human fibroblasts produce fibronectin but do not retain it in the cell membrane. The tumorigenicity of certain virus-transformed cell lines was inversely correlated with the levels of surface-associated fibronectin. Transformed cells thus may be producing factors that help them in destroying fibronectin or in releasing it from their surfaces. We therefore analyzed the supernantant fluids of fibrosarcoma cells (8387) to find activities responsible for the loss of fibronectin in transformation and the malignant, invasive state.

Major Findings:

A sensitive radioimmunoassay, specific for human fibronectin, was developed and used to measure the ability of certain biologically active polypeptides to release fibronectin from cultured human lung fibroblasts into their culture media. Concentrated, serum-free culture supernatant from a human fibrosarcoma cell line was fractionated by gel filtration chromatography in the presence of acetic acid. Various polypeptides with molecular weights between 46,000 and 6,000 were tested for their ability to release fibronectin from cells. The fraction, containing polypeptides with an apparent molecular weight of 10,000, exhibited the ability to rapidly release fibronectin from target cells. The activity could be inhibited by phenylmethyl sulphonylfluoride. Several other hormonal factors, tested in parallel with these

fractions, failed to show this effect. Also, biologically active phorbol esters known to dissociate microfilament bundles were able to release fibronectin from cells, which would suggest that there may be an interaction between microfilaments and cellular fibronectin.

We used fibronectin-releasing, 10,000 molecular weight peptides to study their effects on the cell-free matrices of cultured diploid human lung fibroblasts. The isolated matrices contained fibronectin and procollagen as their major radiolabeled proteins and some as yet unidentified polypeptides. The 10,000 molecular weight peptides released both fibronectin and procollagen from the matrices. After digestion of the matrix-associated collagen with collagenase, the 10,000 molecular weight peptides released only fibronectin. Collagenase treatment, on the contrary, did not affect matrix-collagen with collagenase, the 10,000 molecular weight peptides released only fibronectin. Collagenase treatment, on the contrary, did not affect matrix-associated fibronectin. In addition, a 66,000 molecular weight matrix-associated protein was constantly cleaved to a 62,000 form that remained in the matrix.

The ability of human fibronectin to bind directly to actin was studied by using actin-Sepharose conjugates. A quantitative recovery of soluble human fibronectin could be accomplished by chromatography on actin immobilized on Sepharose beads. Human fibronectin molecules, bound to actin-Sepharose, could be eluted with 0.25-0.35M potassium bromide. Fibronectin isolated by actin-Sepharose chromatography co-migrated in SDS-polacrylamide gel electrophoresis with fibronectin purified by conventional procedures. We have recently found by using proteolytic fragments of chicken cellular fibronectin that there is a specific fragment that binds to actin. This fragment is different from the gelatin (collagen) binding fragment.

Significance to Biomedical Research and the Program of the Institute:

The 10,000 dalton molecular weight polypeptides may represent a family of cellular gene products responsible for maintenance of low levels of surface associated fibronectin in fibrosarcoma cells and thus may be related to their infiltrating properties by preventing the formation of the extracellular matrix. The results of fibronectin releasing protein indicate that the fibronectin releasing peptide behaves as a specific protease on the matrices of cultured human fibroblasts and that there is apparently a direct association between collagen and fibronectin in-vivo. The efficient direct association of fibronectin to actin suggests that interactions between these proteins might also take place in-vivo but further studies are needed to elucidate the biological significance of this affinity. If the interaction between actin and fibronectin plays a role in the normal anchorage, morphology and growth behavior of cells, a modification of either actin or fibronectin might then regulate their mutual interaction. These studies also offer the background for the direct examination of the nature of intermolecular interaction between cytoskeleton and cell surface proteins in normal cells, during different stages in cell cycle and during cellular transformation.

Proposed Course:

This project has been completed.

Publications:

Keski-Oja, J., Marquardt, H., De Larco, J. E., and Todaro, G. J.: Human fibrosarcoma cells produce fibronectin-releasing peptides. J. Supramolec. Struct. 11:217-225, 1979

Keski-Oja, J., Shoyab, M., De Larco, J. E., and Todaro, G. J.: Rapid release of fibronectin from human lung fibroblasts by biologically active phorbol esters. Int. J. Cancer 24:218-244, 1979.

Rapp, U. R., Keski-Oja, J., and Heine, U. I.: Establishment and characterization of the epithelial mouse embryo cell line MMC-E. Cancer Res. 39:4111-4118, 1979.

Vesterinen, E. H., Carson, J., Walton, L. A., Collier, A. M., Keski-Oja, J., Hedrud, J. G., and Pagano, J. S.: Human ectocervical and endocervical epithelial cells in culture: A comparative ultrastructural study. Am. J. Obstet. Gynecol. 1980, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05148-01-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Genetics, Epidemiology, and Natural History of Outbred Colonies of Various Species of Mus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Callahan	Expert	LVC	NCI
OTHER:	G. J. Todaro	Medical Officer	LVC	NCI
	J. Schlom	Microbiologist	LVC	NCI
	U. R. Rapp	Visiting Scientist	LVC	NCI
	E. Kuff	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)
Mr. George Schidlovsky, Brookhaven Laboratories

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Outbred colonies of various Asian species of Mus were used for identification and study of new endogenous retroviral genes and genetic elements involved in the etiology of neoplasia. Specifically, (1) the evolutionary relationship between the different Asian species of Mus represented was established; (2) the colonies were monitored for the development of spontaneous tumors and determinations were made to see whether there is a correlation between expression of endogenous retroviruses and specific types of tumors; and (3) the colonies were maintained as a resource for the identification of new classes of endogenous retroviruses and transformation related genes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To develop outbred colonies of various species of Mus to utilize for the identification and study of new endogenous retroviral genes and genetic elements involved in the etiology of neoplasia. Specifically, (1) to establish the evolutionary relationship between the different Asian species of Mus represented in the colonies; (2) to monitor the colonies for the development of spontaneous tumors and determine whether there is a correlation between expression of endogenous retroviruses and specific types of tumors; and (3) to develop the colony as a resource for the identification of new classes of endogenous retroviruses and transformation related genes.

Methods Employed:

As described in Project Z01-CP-04829-06-LVC several outbred colonies of Asian species of Mus were established to study the genetic variation of endogenous retroviral genes and genetic elements affecting their expression or involvement in the etiology of neoplasia. In concert with these studies, it was deemed necessary to understand the evolutionary relationship between the different Asian species of mice as well as aspects of their natural history relevant to the development of neoplasia. It was therefore of interest to monitor the different colonies for the development of spontaneous tumors with the expectation that in certain cases endogenous retroviral genes might be involved as an etiologic agent in the disease.

Major Findings:

Over the past four years we have successfully established outbred colonies of several species of the genus Mus. Mice of that species obtained from different provinces of Thailand have been maintained as separate colonies. In addition, we are maintaining other species of mice which have not bred. Each of these species is reproductively separate and easily identified by a variety of biochemical, anatomical and karyological traits. The evolutionary relationship between these species was determined by measuring the nucleic acid homology between the nonrepetitive cellular DNA sequences, using as a probe, radioactively labeled nonrepetitive cellular DNA from M. cervicolor cervicolor. By this criteria, M. cervicolor cervicolor, its subspecies M. cervicolor popaeus and M. cookii are the most evolutionarily related. Of the other species tested M. caroli, M. musculus, and M. dunni are more distantly related, while the least related species are M. pahari and M. shortridgei. In other experiments, it was shown that M. shortridgei, nonrepetitive cellular DNA when hybridized to mouse, rat or hamster cellular DNAs, shows more homology to mouse DNA than to either rat or hamster. This is consistent with it being a member of the genus Mus. Thus members of the genus Mus represent a group of species sharing a broad range of nucleic acid

sequence homology between their cellular genomes. The cellular DNA of members of the genus Vandeleuria, as shown by nucleic acid hybridization, share similar levels of nucleic acid sequence homology with cellular DNA from members of the genera Mus and Rattus, suggesting the three genera diverged from a common ancestor.

Since the establishment of the colonies, the incidence of spontaneous tumors in each has been monitored. With one exception, all of the colonies exhibit a frequency of spontaneous tumors of 1.5 percent or less. The average age at which tumors do arise is 18 to 25 months. However, one M. cervicolor popaeus colony, begun with mice from the Tak province of Thailand, exhibits a 3.3 percent incidence of spontaneous tumors. Moreover, half of these tumors were mammary adenocarcinomas. By tracing the ancestry of these mice, within the colony, it was determined that all were descendents of one of 3 of the original females breeders, 2 of which had also developed mammary tumors and that the development of mammary tumors could not be associated solely with their paternal ancestry. The frequency of mammary tumors in females of these 3 lineage groups is 20 to 30 percent. Many of these tumors were pregnancy dependent in their development, although mammary tumors have also developed in nonparous females. The age range during which tumors developed is 7 to 33 months. In one lineage group, mammary tumors were observed in 4 successive generations, the average age at which tumors were initially observed is 16 months. Many of the initial mammary tumors were analyzed by electron microscopy for the presence of retroviruses. In some cases, type-B viruses were observed in mammary tissue. In collaboration with Dr. J. Schlom, NCI, the milks of mice in this colony was found to contain type-B retroviral particles that were morphologically indistinguishable from those observed in the M. cervicolor popaeus mammary tumors and MMTV of M. musculus.

The M. cervicolor popaeus milk borne type-B viral particles were purified and used to prepare antisera to the viral associated proteins. Using this antisera, as well as, antisera to M. musculus MMTV, it was shown, in competition radioimmunoassays, that the milk-borne virus is easily distinguished from laboratory strains of MMTV. This excludes the possibility, therefore, that the milk borne virus is the result of a trans-species transmission of MMTV from M. musculus into the the M. cervicolor popaeus colony. The availability of these new immunological reagents has led to the development of interspecies radioimmunoassays and immunoperoxidase assays for type-B viral proteins. Using these assays, the expression of type-B viral proteins has been detected in the milk of other murine species including M. cervicolor cervicolor, M. caroli and M. cookii. In each of these species the type-B viral proteins expressed were immunologically distinguishable from laboratory strains of M. musculus MMTV.

We tested protein extracts of the M. cervicolor popaeus mammary tumors for the expression of endogenous retroviruses (type-B, type-C-I, type-C-II, and M432) using competition radioimmunoassays (see Project Z01-CP-04829-06-LVC). Significantly, 85% of the tumors tested (18/21) were positive for the expression of type-B viral proteins. Other tissues from some of these tumored

animals were tested and found to be negative for type-B viral expression. Similar results were obtained using antisera to the M. cervicolor popaeus milk-borne type virus in an immunoperoxidase assay. Type-C-I viral proteins were expressed in 5 of the 21 tumors tested, whereas none were positive for the expression of type-C-II or M432 viral proteins. The consistent observed expression of the type-B virus in these tumors, but not in other tissues, strongly implicates this class of viruses in the etiology of the disease. Currently, the expression of type-B virus is being correlated with the histological characteristics of these tumors.

One of the striking features of these high incidence lineage groups is that the manner in which mammary tumors develop seems to represent a composite of what has been observed in different inbred strains of M. musculus. In an effort to dissect the apparent complex of factors involved in the development of the disease, we have initiated inbreeding experiment with selected mice of this colony to enrich for the development of pregnancy dependent and pregnancy nondependent tumors. In other experiments, we are attempting to transmit potential oncogenic viruses from extracts of M. cervicolor popaeus mammary tumors to BALB/c and C57BL/6 inbred strains of M. musculus and M. cervicolor mice which are free of mammary tumors in their ancestry.

In addition to mammary tumors we have focused on spontaneous lymphosarcomas in the different colonies of mice. The incidence is low, less than 0.1%, and the tumors were observed at 18 to 24 months. However, we have recovered two type-C viral isolates from lymphosarcomas in M. cervicolor cervicolor and M. cervicolor popaeus which induce this disease with 10% of inoculated NIH Swiss or M. cervicolor mice. One of the viral isolates (M813) has been studied in more detail. M813 is a type-C-II endogenous retrovirus (see Project ZO1-CP-04829-06-LVC) which replicates to high titer in tissue culture cells derived from both M. musculus and M. cervicolor. Nucleic acid hybridization studies have shown that the M813 viral genome shares partial sequence homology with other C-II viruses isolated from M. cervicolor cultured cells. Sequences related to the viral genome are poorly represented in DNA of M. musculus. Recently, in collaboration with Dr. U. Rapp, an endpoint diluted stock of the M813 virus was injected into NIH Swiss mice. Within four months one of these mice developed an ovarian carcinoma. Extracts of this tumor were also injected into NIH Swiss mice. Currently 20% of these latter mice have developed leukemia within three months. Currently we are attempting to dissect the genomic changes, which must have occurred in the development of the rapid leukemia virus. If recombinational events with the cellular DNA of NIH Swiss mice have played a role in this change, we should be able to identify the new sequences since the M813 viral genome shares little homology with cellular DNA of M. musculus. Employing a similar logic reciprocal, experiments have been undertaken using M. musculus type-C viruses and M. cervicolor mice.

Significance to Biomedical Research and the Program of the Institute:

We have established several colonies of an evolutionary diverse group of

species of the genus Mus which are not commonly available in this country. The availability of these colonies has led to the identification and characterization of two new classes of endogenous retroviruses, thus adding a new dimension to previous work on endogenous retroviruses of M. musculus. The low incidence of spontaneous tumors in these colonies suggests that they will be useful in future studies directed at the retrieval of induced transformation specific genetic elements. The identification of lineage groups of M. cervicolor popaeus which have a high incidence of mammary tumors present an unique alternative to inbred strains of M. musculus for studies of the etiology of this disease. Moreover, the identification of type-B virus in tumors and milks of some of these species is the first such demonstration in a species other than M. musculus and has led to the development of the first interspecies radioimmunoassays for type-B viral proteins.

Proposed Course:

The present project will be continued and extended to complete the objectives described above.

Publications:

Horan Hand, P., Teramoto, Y. A., Callahan, R., and Schlom, J. Interspecies radioimmunoassay for the major internal protein of mammary tumor viruses. Virology (in press).

Teramoto, Y. A., Horan Hand, P., Callahan, R., and Schlom, J. Interspecies immunoassays detect novel mammary tumor viruses. J. Natl. Cancer Inst. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05149-01-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Mechanisms of Tumor Production Growth Factors as Mediator of Tumor Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. E. Shoyab	Expert	LVC	NCI
OTHER:	G. J. Todaro	Medical Officer	LVC	NCI
	J. Keski-Oja	Visiting Fellow	LVC	NCI
	J. E. De Larco	Expert	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project aims to isolate and characterize putative endogenous tumor-promoters and to develop a rapid and economical assay for identification of exogenous and endogenous tumor-promoters. The following activities were specifically pursued: (1) To elucidate the mechanism of TPA induced inhibition of EGF binding (TIEB). To search for compound(s) which can reverse and modulate TIEB at non-toxic doses. (2) To isolate and characterize membrane receptors of phorbol esters. (3) To search for endogenous ligand(s) for phorbol receptors, isolate and characterize them. (4) To isolate and characterize growth factor(s) induced by biologically active phobol esters. (5) To test whether EGF and other growth factors enhance the carcinogenesis in-vivo and in-vitro. (6) To investigate whether TPA induces or enhances the expression of endogenous oncogenic cellular information. (7) To study whether retroviruses act as promoters in the carcinogenesis started by chemical or physical agents. (8) The reversal of anchorage independent growth of transformed cells by differentiation inducing agents.

Project Description

Objectives:

The process of tumor-induction has been broadly dissected into two stages, i.e., initiation and tumor promotion. The initiation process apparently involves irreversible alteration in the genetic material whereas tumor promotion appears to be epigenetic in nature and is reversible at least in the early stages. Hence, the interruption of carcinogenic process should be feasible at the tumor promotion stage. This project aims to isolate and characterize putative endogenous tumor-promoters and to develop a rapid and economical assay for identification of exogenous and endogenous tumor-promoters. The following activities were specifically pursued: (1) To elucidate the mechanism of TPA induced inhibition of EGF binding (TIEB). To search for compound(s) which can reverse and modulate TIEB at non-toxic doses. (2) To isolate and characterize membrane receptors of phorbol esters. (3) To search for endogenous ligand(s) for phorbol receptors, isolate and characterize them. (4) To isolate and characterize growth factor(s) induced by biologically active phobol esters. (5) To test whether EGF and other growth factors enhance the carcinogenesis in vivo and in vitro. (6) To investigate whether TPA induces or enhances the expression of endogenous oncogenic cellular information. (7) To study whether retroviruses act as promoters in the carcinogenesis started by chemical or physical agents. (8) The reversal of anchorage independent growth of transformed cells by differentiation inducing agents.

Methods Employed:

Tumor promoters are a specific class of co-carcinogens which themselves are noncarcinogenic but which can induce tumors in animals previously treated with a suboptional dose of certain chemical carcinogens. Among the most potent of these compounds are plant diterpenes including 12-O-tetradecanoyl-phorbol-13-acetate (TPA). TPA and other biologically active phorbol esters elicit and modulate a variety of biochemical and biological functions in mouse skin including stimulation of macromolecular synthesis, histone phosphorylation, synthesis of phospholipids and modulation of the metabolism of polyamines and cyclic nucleotides. In addition, these compounds induce ultrastructural changes in and affect the differentiation of murine epidermis. Tumor-promoting phorbol esters also evoke pleiotypic responses in cultured cells, including stimulation of macromolecular synthesis and cell proliferation, induction or plasminogen activator and ornithine decarboxylase, loss of surface-associated fibronectin, alterations in the metabolism of cyclic nucleotides and polyamines, stimulation of prostaglandin synthesis, either the inhibition or stimulation of differentiation, alterations in cell morphology and permeability, and elevation in the level of Na^+-K^+ ATPase activity.

Several biochemical and biological studies provide evidence that the initial site of action of tumor-promoting phorbol esters may be the membrane of target cells. The tumor-promoting phorbol esters have been found to modulate the interaction between epidermal growth factor (EGF) and its membrane receptors in a variety of cells in culture. The pleiotypic effects of TPA and the related tumor promoters in-vivo as well as in-vitro mimic several actions of growth stimulating polypeptide hormones such as EGF and sarcoma growth factor (SGF). We have formulated as working hypothesis, that TPA and certain analogues may have some structural resemblance to the endogenous growth promoting and differentiation modulation substance(s) that have specific membrane receptors. TPA recognizes and interacts with these receptors, thus mimicking the action of putative substance(s). Furthermore, TPA can induce and regulate the production of these endogenous substance(s). TPA can also increase cell susceptibility to a normal regulator of cell growth and modulator of cell differentiation.

Major Findings:

A. Affinity Modulation of Epidermal Growth Factor Membrane Receptors by Biologically Active Phorbol Esters.

TPA (12-O-tetradecanoyl-phorbol-13-acetate) reversibly inhibits binding of ^{125}I-labeled epidermal growth factor (EGF) to treated normal mouse, human, mink, cat, rabbit, hamster, rabbit and chicken cell lines as well as DNA virus transformed or chemically transformed murine cells. TPA does not affect the binding of concanavalin A (Con A), multiplication stimulating activity, (MSA) insulin, murine retrovirus glycoprotein (gp70), nerve growth factor (NGF), alpha-2-macroglobulin (-2-M) and low density lipoprotein (LDL) to their respective membrane receptors. It alters the affinity of the receptors for EGF without changing the total number of EGF receptors per cell. The scatcard plots of EGF binding gave curvilinear plots in all the cells investigated suggesting either the heterogeneity of receptor population or the negative cooperativity. TPA inhibition of EGF binding (TIEB) is a temperature, dose and time dependent phenomena. TIEB is completely abolished at 4^{o}C. TIEB is also not exhibited with fixed cells or isolated plasma membrane. Thus, it appears that TIEB is related to the membrane fluidity and those treatments which affect the transmembrane mobility of receptors also alter the TIEB. A puzzling feature of TIEB is that if cells are continuously exposed to TPA for a longer time the degree of TIEB starts to gradually decrease after a few hours and cells become almost refractory after a few days. This loss of TIEB is not due to conversion of TPA to an inactive form because if TPA containing media from these cells is transferred to new cells, a 85-90% inhibition of EGF binding is observed.

TIEB is not significantly affected by steroid hormones, nonsteroid anti-inflammatory drugs, mouse interferon, cytoskeleton disrupting agents, modulators of polyamine metabolisms, protease inhibitors, inhibitors of DNA, RNA

and protein synthesis, by various mucopolysaccharides, water soluble vitamins, vitamins D, E and K1. However, it is partially reversed by retinoids and oubain (Na^+-K^+ ATPase inhibitors). Those phorbol esters (Tigliane, Ingenane and Daphnane Types) which stimulate cell growth in culture and have tumor promoting activity in-vivo in two stages tumorigenesis model alter the EGF receptor affinity, while the biologically inactive derivatives fail to change the affinity of EGF receptors interaction. Rapid assays are needed for tumor promoting agents both of exogenous and endogenous origin. The specificity, sensitivity and rapidity of TIEB might provide a means for the qualitative and quantitative detection of other classes of tumor promoting agents.

B. Perturbation of Membrane Phospholipids Alters the Interaction Between Epidermal Growth Factors and its Membrane Receptors.

To determine the chemical environment of EGF-receptor sites in the plasma membrane and the role of cell membrane lipids, we investigated the consequence of the exposure of cells in culture to the various hydrolytic enzymes and certain lipid disrupting agents on EGF-receptors interactions. Phospholipase C, like the tumor-promoting phorbol esters, specifically inhibits the binding of ^{125}I-labeled epidermal growth factor (EGF) to treated mink lungs and murine 3T3 cells but does not reduce the binding of various other ligands to their membrane receptors. Phospholipase C digestion decreases the affinity of the receptors per cell. Certain other agents that interact with membrane phospholipids mimic the phospholipase C effect. The results suggest a role for membrane phospholipids in the EGF-receptor interaction and TIEB may involve the perturbation of the organization of membrane phospholipids.

C. Vitamin K3 and Related Quinones Modulate the Affinity of Epidermal Growth Factors for its Receptors.

The effects of vitamin K3, quinones fat soluble vitamins, and various naturally occurring and synthetic compounds on the binding of ^{125}I-EGF to mink lung cells or murine 3T3 cells in culture was studied. Vitamin K3 but not other fat soluble vitamins markedly inhibits the binding of ^{125}I-labeled EGF to treated cells, but does not affect the binding of insulin, concanavalin A (Con A), alpha-2-macroglobulin and murine leukemia virus glycoprotein, (gp70), to their membrane receptors. The binding of multiplication stimulating activity (MSA) to treated cells is also reduced to some extent. Vitamin K3 alters the affinity of the receptors for EGF without changing the total number of available receptors per cell. Vitamin K3 modulation of EGF-receptor interaction is a temperature, dose, and time dependent phenomena. EGF-receptor interaction is also significantly modulated by 1, 4-napthoquinone, 1-4, benzoquinone and phenanthrenequinone but not by other quinones of anthracyclin antibiotics. Experiments are in progress to test whether vitamin K3 and related quinone act as tumor-promoters.

D. Cell Membrane Receptors for Phobol Esters.

A variety of fibroblastic as well as epithelioid cells contain site(s) on their membrane which specifically interact with the tumor-promoting and some of the the non-tumor-promoting derivatives of phorbol. The binding of labeled TPA (12-O-tetradecanoyl-phorbol-13-acetate) to live on glutaraldehyde-fixed cells is time- and temperature-dependent and can be specifically inhibited by excess unlabeled TPA as well as all of the biologically active derivatives of TPA, but not by the biologically inactive derivative 4-O-methyl TPA (Me-TPA). Retinoic acid also inhibits the interaction between phorbol esters and their receptors while epidermal growth factor (EGF) has no effect. The isolation and characterization of EGF receptors should provide useful information toward understanding the intricate process of tumor promotion.

E. Rapid Release of Fibronectin from Human Lung fibroblasts by Biologically Active Phorbol Esters.

A sensitive radioimmunoassay technique has been used to study the effects of several phorbol esters on their ability to release fibronectin from cultured human lung fibroblasts into medium. The biologically active phorbol esters studied rapidly released fibronectin from cells in medium, with concomitant changes in the cellular morphology within two hours. The quantity of fibronectin released was dose-, time- and promoter-dependent. The earliest release of fibronectin was seen within 30 minutes of onset of the incubation. Alterations in membrane topology elicited by phorbol esters appear to be responsible for the rapid release of fibronectin molecules from cells into the medium.

Significance to Biomedical Research and the Program of the Institute:

The EGF-competing activity of the phorbol esters parallels the tumor-promoting activity in-vivo. The phorbol derivatives lacking tumor-promoting activity also lack EGF-competing activity. TPA treatment seems to modulate EGF binding by decreasing the affinity of the receptors on the treated cells for EGF, rather than decreasing the number of receptors per cell. This affinity modulation is reversible and dependent on time, temperature and TPA concentration. The effect appears to be specific, for the EGF receptor system, as four other receptor-ligand systems tested in the same TPA-treated cells and three receptor-ligand systems in the other cells did not show any alterations in receptor affinity. TPA modulation of EGF binding is observed with doses of promoter comparable to those required to elicit biological response in-vivo as well as in-vitro. The above data suggests that TPA-mediated alterations in growth factor(s)-receptor interactions might be related to the underlying mechanism by which tumor-promoting agents initiate a chain of events causing alteration in cellular growth and function. Interestingly, EGF has been reported to enhance the tumorigenesis induced by chemical carcinogen. SGF, produced by mouse sarcoma virus-transformed cells also inter-

acts with EGF receptors, stimulates cell growth and anchorage-independent growth. The putative endogenous growth factor(s) produced in response to the exposure of cells to tumor-promoting agents, may, then, activate a program of gene expression in those cells that have already been genetically altered by initiating agents.

Proposed Course:

This project will continue and extend the findings described to better define the mechanisms of tumor promotion. There is need for rapid cell culture assay for tumor-promoting agents both of exogenous and endogenous origin. The data presented here, showing the consequences of promoter treatment on EGF-receptor interactions and the specificity, sensitivity and rapidity of this response, might provide a means for the qualitative and quantitative detection of other classes of tumor-promoting agents. The isolation, characterization and mode of action of putative endogenous growth and differentiation modulating substances (endogenous tumor-promoter(s)) should provide useful information in understanding the mechanism of growth, differentiation and carcinogenesis. These studies have potential to provide clues in designing strategies to reverse and suppress the process of neoplastic development.

Publications:

Shoyab, M., De Larco, J. E., and Todaro, G. J. Biologically active phorbol esters specifically alter affinity of epidermal growth factor membrane receptors. Nature 279:387-391, 1979.

Keski-Oja, J., Shoyab, M., De Larco, J. E., and Todaro, G. J. Rapid release of fibronectin from human lung fibroblasts by biologically active phorbol esters. Int. J. Cancer 24:218-224, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05150-01-LVC

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
New Approaches to the Study of Chemical Carcinogenesis and Co-carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	U. R. Rapp	Visiting Associate	LVC	NCI
OTHER:	G. J. Todaro	Medical Officer	LVC	NCI
	U. Heine	Microbiologist	LVC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Improved in-vitro assays for chemical transformation of fibroblastic and epithelial cells was developed and co-carcinogenesis was used for the isolation of new transforming type-C viruses. Specifically: (i) Non-transformed, syngeneic epithelial and fibroblastic mouse embryo cell lines susceptible to chemical carcinogenesis in-vitro were established. Assay systems were developed to allow processing large numbers of carcinogen treated cells and performance of transformation experiments in a shorter period of time. (ii) Co-carcinogenesis was used for the isolation of efficiently transforming type-C viruses in culture. (iii) Growth promoting factors and transforming factors from chemically transformed epithelial cells were isolated by the use of nontransformed epithelial cells as test cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

Development of improved in-vitro assays for chemical transformation of fibroblastic and epithelial cells. Use of co-carcinogenesis for the isolation of new transforming type-C viruses. Specifically: (1) Develop cell substrates for chemical transformation of a variety of differentiated cells in-vitro. (2) Improvement of assay systems to allow processing large numbers of carcinogen treated cells and development of transformation experiments in a shorter period of time. (3) Use of co-carcinogenesis for the isolation of efficiently transforming type-C viruses in culture. (4) Isolation of growth promoting and transforming factors from chemically transformed epithelial cells using non-transformed epithelial cells as on test cells.

Methods Employed:

During the past ten years several lines of evidence have developed to indicate a possible interaction between chemical carcinogens and endogenous RNA viruses. Most of this work has been done in-vivo where it is difficult to sort out the relative contributions of transformation frequency versus the hosts immune response to the overall tumor incidence. We have therefore concentrated on developing quantitative in-vitro systems for chemical carcinogenesis. Since most human neoplasia arise in epithelial tissues, emphasis was placed on establishing epithelial cells. Drawbacks of chemical transformation assays in-vitro are the required large size of the experiments (low transformation frequencies) and the long time (4-6 weeks for completion). We developed conditions which allowed assay of large numbers of treated cells within 1 to 2 weeks.

To facilitate the generation of new transforming type-C viruses both in-vivo and in-vitro we examined the effect of carcinogen treatment. Based on our previous observations that carcinogen/mutagen treatment in-vivo occasionally shortened the latency period with which C3H MuLV induced tumors, such tumors were established in vitro and type-C virus isolations techniques were applied with good results.

Major Findings:

A. Establishment of non-transformed, syngeneic epithelial and fibroblastic mouse embryo cell lines susceptible to chemical carcinogenesis in-vitro.

A fibroblastic (MMC-F) and an epithelial (MMC-E) cell lines were established from a single embryo of the wild mouse strain Mus musculus castaneous (Rapp et. al., Cancer Res. 39:4111, 1979). Clonal derivatives of these lines have been grown for many serial passages without any significant morphological changes. These cell lines do not express antigens related to known mouse

type-C viruses and are resistant to activation by bromodeoxyuridine. We have classified the MMC-E cell line (clone 7) as epithelial by the following criteria: (a) morphological appearance, (b) low levels of extracellular fibronectin, and (c) the demonstration of tight junctions and desmosomes at high resolution. MMC-F and MMC-E cells form flat monolayers in plastic dishes and do not grow in soft agar; tests for tumorigenicity of cells at all passages were negative. Both cell lines are susceptible to chemical as well as type-C viral transformation in-vitro. Inoculation of transformed MMC-F cells into nude mice gave rise to fibrosarcomas, whereas transformed MMC-E cells produced poorly differentiated carcinomas. The availability of a set of stable, non-transformed syngeneic mouse cell lines which differ in their phenotype should prove useful for studies on the mechanisms of malignant transformation. The epithelial cell line, MMC-E, in particular, should be helpful in the study of chemical as well as viral carcinogenesis in view of the fact that the majority of human tumors are of epithelial origin, and an appropriate epithelial cell culture system has thus far been unavailable.

B. Use of the epithelial and fibroblastic mouse cell lines MMC-E/MMC-F for the determination of the target cell specificity of sarcoma growth factor (SGF).

The effects of epidermal growth factor (EGF) on the growth and morphology of mouse embryo epithelial cells (MMC-E) were studied in culture. Growing cultures of epithelial cells were incubated in the media containing EGF or certain other mitogenic peptides. It was found that nanogram quantities of EGF stimulated growth in these cells and caused reversible phenotypic changes in these cells. These changes were not observed in cultures treated with the other mitogens. The compact growing islands of MMC-E cells were surrounded by elongated border cells (Rapp et al., Cancer Res. 39:4111, 1979). EGF induced the elongated border cells to flatten and spread. The change of the elongated border cells into polygonal, flattened cells was dependent on the dose of EGF. After treatment with higher concentrations of EGF all cells appeared more flattened and their cytoplasm was more granular than that of the controls. Scanning electron microscopic studies showed that the elongated border cells in the control cultures were distinctly higher than the cells inside the islands, while after exposure to EGF they flattened and had fewer surface microvilli than control cells. When EGF was removed and the cells were further cultivated in media without EGF, the border cells became smaller and elongated, eventually resembling those in the control cultures. These results show that EGF may act as a regulatory factor in the control of the proliferation and differentiation of mouse epithelial cells. (Keski-Oja et al., Exp. Cell Res. 128, in press, 1980.)

C. Effect of Murine Sarcoma Growth Factor (SGF) on the Growth and Morphology of Cultured Mouse Epithelial Cells.

The MMC-E cell line was also used to study the effects of purified murine sarcoma growth factors (SGFs) on the growth of epithelial cells. Murine SGF

was a partially purified preparation from the serum-free culture media of mouse fibroblasts transformed by Moloney murine sarcoma virus. SGF stimulated DNA-synthesis in resting cells and induced them to grow to higher densities than control cells. With continued exposure to SGF, MMC-E cells lost the postconfluency inhibition of division and formed expanding foci. When the SGF was removed and the cells were subcultured, they regained their normal phenotype showing that the effects of SGF are reversible for these cells. SGF could also stimulate these cells to grow in soft agar, like the syngeneic fibroblasts (MMC-F) from the same mouse embryo, but slower than the control fibroblastic clone. Microgram quantities of SGF were needed to stimulate soft agar growth of MMC-E cells. These results indicate that SGF can bring about a phenotypic change in the growth pattern of epithelial cells as well as fibroblastic cells. (Keski-Oja et al., J. Cell. Physiol. 103, in press, 1980.)

Significance to Biomedical Research and the Program of the Institute:

The overall goal of this work is to characterize cellular genes that are associated with the induction of cancer as it occurs spontaneously or after induction with carcinogens. Considering the fact that carcinomas are especially prevalent in humans, we have recently put special emphasis on transformation of epithelial cells. A family of cell-derived transforming genes isolated as part of type-C viruses might provide the tools necessary for both an understanding of mechanisms of transformation, and the development of strategies to neutralize their action.

Proposed Course:

Recent evidence from transfection experiments with chromosomal DNA has shown (Chiaho et. al, Proc. Natl. Acad. Sci. (USA) 76:5714, 1979) that chemical transformation of cells may be achieved by the activation of single tumor genes. These genes may be altered as a consequence of carcinogen treatment or may represent derepressed normal genes. Those chemically transformed cells that were efficient donors of transforming DNA will be tested for their effectiveness as target cells for the generation of new transforming retroviruses. It appears that both DNA transfection and type-C virus mediated transduction will lend themselves to the study of genetic changes related to chemical carcinogens.

Publications:

Rapp, U. R., Keski-Oja, J. and Heine, U.: Establishment and characterization of the epithelial mouse embryo cell line, MMC-E. Cancer Res. 39:4111-4118, 1979.

Keski-Oja, J., De Larco, J. E., Rapp, U. R. and Todaro, G. J.: Murine sarcoma growth factors affect the growth and morphology of cultured mouse epithelial cells. J. Cell. Physiol. 103: 1980, (in press).

Keski-Oja, J., Keine, U. I., Wetzel, B. and Rapp, U. R.: Epidermal growth factor-induced alterations in proliferating mouse epithelial cells. Exp. Cell Res. 128: 1980, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-CP-05153-01-LVC

PERIOD COVERED

September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)

Analysis of the Biological Activity of Cloned Recombinant MSV DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. D. G. Blair, Expert Consultant	LVC, NCI
P. J. Fischinger, Head, Virus Control Section	LVC, NCI
W. L. McClements	LMV, NCI
M. K. Oskarsson	LMV, NCI
W. G. Robey	LMV, NCI
G. F. VandeWoude, Head, Virus Tumor Biochemistry Section	LMV, NCI

COOPERATING UNITS (if any)

LMV, NCI

LAB/BRANCH Laboratory of Viral Carcinogenesis

SECTION Virus Control Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recombinant DNA clones of the whole and partial genomes of two strains of Moloney MSV, as well as the MSV related normal cell sarc sequence of Balb/c mice, have been isolated and characterized biochemically. We are attempting to define the role of specific regions of the MSV genome in the transformation process by measuring the ability of portions of the MSV genome to transform normal mouse cells in a DNA transfection assay. We are also trying to determine the specific events required to enable normal cell src sequences to express a transforming function.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To define the minimal genetic unit necessary for MSV transformation and to identify the function of specific portions of the genome.

Major Findings:

Whole cloned MSV proviral DNA transformed cells in culture with high efficiency ($\sim 6 \times 10^4$) foci/picomole DNA). Both indirect immunofluorescence and direct immunoprecipitation indicated that cells transformed by the m1 MSV proviral DNA were able to translate the MSV coded p60 precursor polyprotein. The MSV genome present in these cells was also rescuable following superinfection with replicating helper leukemia viruses in most of the single focus isolates tested. Furthermore, infection of transfected cells with optimal amounts (m.o.i. ~ 0.1) of helper MLV resulted in the appearance of early, helper dependent foci approximately 5-7 days later, while in the absence of helper, foci did not appear until 10-15 days after transfection. The helper dependent assays indicated that transfection with whole MSV proviral genomes produced 50,000-100,000 foci per µg MSV DNA.

Subgenomic, src containing fragments from either the 3' or 5' portion of the MSV genome also transformed efficiently, although the specific infectivity was reduced 6-fold. The 3' src-containing fragment pm13 was not rescuable, but a small amount of infectious transforming virus could be rescued from cells transfected with fragments containing src and the 5' end of the MSV genomes.

Internal fragments lacking either copy of the terminal redundancy (TRS) transformed with low efficiencies. A detailed comparison of the sequences present in the various transforming fragments and analysis of clones constructed by linking the internal src-containing, poorly transforming fragments to non-transforming sequences carrying the TRS, strongly suggested that the presence of one copy of the 0.6 kb TRS from either the 5' of the 3' end of the genome increased the efficiency of cell transformation about 1000-fold. Furthermore, cotransfection of cells with separate individual cloned fragments containing the terminal redundancy and src also resulted in enhanced transformation. Normal cellular mouse "sarc" was linked to sequences derived from the 5' end of MSV, including one copy of TRS, and cloned in vitro, the new structure was able to transform with efficiencies similar to those observed with MSV src. This formation might have recapitulated the original recombinational event which generated MSV.

Significance:

Isolated presumptive tumor virus genes cloned bacteria and proven to be biologically functional is an obvious approach to dissecting the mechanism, regulation and sequences involved in the expression of viral transforming genes. The identification of specific, non-transforming regions of the viral genome which "turn-on" previously inactive sequences to an active transforming state should provide valuable insights into how other non-transforming sequences present in normal cells are activated to induce cell transformation.

Proposed Course:

Further analysis of permutations of cloned DNA's of the MSV src, TRS, and MuLV derived regions will unequivocally establish those nucleotide sequences critical to transformation. Ongoing parallel investigations of the above will sequence critical DNA sites relative to promoter and operator activities. Cells transformed with a single TRS-src derivative will be examined as well as the nature and content of flanking cellular sequences. The role of TRS in activating these cloned genes will be examined. Attempts will be made to isolate and clone non-viral transforming sequences from spontaneous and chemically transformed cell in order to compare these sequences with the model MSV src system.

Publications:

Blair, D. B., McClements, W. L., Oskarrson, M. K., Vande Woude, G. F., and Fischinger, P. J.: Recombinant DNA cloning of murine oncornaviruses: Role of defined sarcoma and leukemia genetic sequences in transformation. In Yohn, D. S., Lapin, B. A. and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research 1979. New York, Elsevier/North-Holland, 1979, p. 137.

Blair, D. G., McClements, W. L., Oskarsson, M. K., Fischinger, P. J., and Vande Woude, G. F.: The biological activity of cloned Moloney sarcoma virus DNA - Terminally redundant sequences may enhance transformation efficiency. Proc. Natl. Acad. Sci. USA, in Press, 1980.

Oskarsson, M. K., McClements, W. L., Blair, D. G., Maizel, J. V., and Vande Woude, G. F.: Properties of a normal mouse cell DNA sequence (sarc) homologous to the src of Moloney sarcoma virus. Science 207: 1222-1224, 1980.

Vande Woude, G. F., Oskarsson, M. K., Blair, D. G., Fischinger, P. J., and McClements, W. L.: Properties of two regions of the Moloney leukemia virus genome required for transformation by src/sarc. In: Animal Viruses. Proceedings of the 1980 ICN-UCLA Symposia on Molecular and Cellular Biology. 2. Animal Virus Genetics. Keystone, Colorado, March, 1980.

Vande Woude, G. F., Oskarsson, M. K., McClements, W. L., Enquist, L. W., Blair, D. G., Fischinger, P. J., Maizel, J. V., and Sullivan, M.: Characterization of integrated Moloney sarcoma proviruses and flanking host sequences cloned in bacteriophage lambda. In The Transformed Cell, Cold Spring Harbor Symp. Quant. Biol. Vol 44. In Press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05154-01-LVC

PERIOD COVERED
September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)

Integration of Baboon Type C Virus in Human X Rodent Hybrid Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. Dr. R. Lemons Geneticist LVC, NCI
Dr. C. J. Sherr, Head, Viral Pathology Section LVC, NCI

COOPERATING UNITS (if any)
Dr. Craig Cohen, Tulane University School of Medicine, New Orleans, LA

LAB/BRANCH Laboratory of Viral Carcinogenesis

SECTION Viral Pathology Section

INSTITUTE AND LOCATION Division of Cancer Cause and Prevention
NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Somatic Cell Hybrids derived by fusion of either mouse or hamster cells with human cell lines were infected with baboon endogenous type C virus (BEV) and scored for virus production. The human and rodent chromosomal content of hybrid cells was identified using asays for 30 gene-enzyme systems (isozymes) representing 22 human chromosomes. A concordance between BEV expression and the presence of human chromosome six was observed (97% synteny in 160 analyzed clones) defining a new genetic locus (Bevi) required for productive BEV infection. Southern blot analyses of integrated proviral DNA in hybrid clones showed that (i) cells segregating human chromosome six also segregated the integrated BEV provirus and (ii) BEV is integrated at random molecular sites in human DNA. Thus Bevi is either a preferred complex locus for integration of BEV in human DNA or, alternatively, is a regulatory gene providing functions critical for maintaining the integrated BEV provirus.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

To characterize a new genetic locus (Bevi) in human cells which controls the replication of baboon endogenous type C virus. To distinguish whether the Bevi locus is a site of preferred proviral integration on human chromosome six or whether it controls the stable integration of the baboon provirus at other genetic loci.

Methods Employed:

Somatic cell hybrids between human and rodent cells were prepared by standard cell fusion techniques and were studied for human chromosomal content by trypsin G banding, and by electrophoretic separation and histochemical resolution of human isoenzyme markers. Cells were infected with baboon type C virus (BEV) either before or after fusion. The DNA of hybrid cells was extracted and studied by liquid phase (Cot analyses) or solid phase (Southern blots) hyridization techniques using [^{32} P]-labeled cDNA transcripts complementary to the complete BEV genome. Unintegrated viral DNA intermediates were obtained by a modified Hirt procedure and similarly analyzed. Circular DNA intermediates were digested with Eco RI (single cut), inserted into a lambda bacteriophage vector, and recombinant phages containing a permuted BEV provirus were obtained using standard recombinational DNA procedures.

Major Findings:

Somatic cell hybrids derived by fusion of either mouse or hamster cells with several human parents were infected with BEV and were scored for virus production. The human and rodent chromosomal content of hybrid cells was identified using assays for 30 gene-enzyme systems (isozymes) representing 22 human chromosomes. Each hybrid clone segregated a different subset of human chromosomes but retained the entire rodent genome. A concordance between BEV expression and the presence of human chromosome 6 was observed (97% synteny in 160 analyzed clones). The loss of chromosome six was associated with the absence of viral expression, showing that the genetic function being assayed was post-penetrational and pretranslational in character. These studies defined a new genetic locus (Bevi for BEV infection). In subsequent experiments, somatic cell hybrids derived from a cloned preinfected human parent were scored for integrated viral gene sequences using liquid hybridization techniques. Chromosome six-positive clones contained the provirus (1-2 copies per haploid genome) while chromosome six-negative clones lacked proviral DNA sequences. Chromosome 19 has also been demonstrated to code for a viral cell surface receptor not encoded by the hamster parent, but expressed in mouse parental cells. No other human chromosome was identified which was required for BEV infection.

The molecular sites of BEV proviral insertion in human cells have been studied by restriction enzyme analysis and the Southern blot technique using [^{32} P]-labeled cDNA transcripts complementary to BEV. The DNA of infected human cells has been restricted with Xho I, Eco RI, and Hind III; Xho I recognizes cleavage sites at both BEV proviral DNA termini, while Eco RI and Hind III recognize single sites in proviral DNA. For each copy of integrated provirus, either of the latter two enzymes yields two fragments containing proviral DNA linked to host cellular sequences. Thus, distinct sites of BEV integration can be distinguished by adventitious sites of Eco RI or Hind III cleavage in flanking host cellular DNA. Studies with single cell clones of infected human cells showed that clones derived from different infectious events contained proviral DNA copies at different molecular sites. The DNA of preinfected human VA-2 x hamster BHK hybrid clones, many of which were previously analyzed for BEV DNA sequences by liquid hybridization techniques, has been extracted, digested with Xho I, and similarly studied. As expected, a single proviral DNA fragment (8.5 kbp) was detected in all chromosome 6+ clones but was absent from chromosome 6- segregants. Results using Eco RI show that counterselected chromosome 6+/chromosome 19+ hybrid clones do not maintain integrated BEV copies at the original sites of parental proviral DNA insertion while counterselected chromosome 6+/chromosome 19- segregants retain provirus only at parental integration sites.

Significance to Biomedical Research and the Program of the Institute:

This system affords the possibility of identifying human genes with novel functions that influence viral integration. That such genes could be present raises the possibility that their primary role is to regulate non-viral genetic elements in human cells.

Proposed Course:

If chromosome 6 contains a preferred molecular integration site for BEV (Model I), we would have expected to observe the same Eco RI and Hind III viral-containing fragments in hybrid clones as those detected in the infected VA-2 human parental clone. However, since the integration sites in hybrid clones were found to differ from parental proviral insertions, the possibility that stable proviral integration requires a chromosome six-coded maintainance function would be considered (Model II). In the latter case, segregation of chromosome six would be expected to result in the concommitant loss of proviral DNA copies integrated anywhere in the human genome. By contrast, segregation of a chromosome containing the BEV provirus could lead to reintegration of BEV at other molecular loci in chromosome six-positive cells. Because segregation of chromosomes in hybrid colonies occurs randomly, and because hybrids undergoing chromosome loss are heterogeneous with respect to any particular chromosome, reintegration in virus-negative segregants could occur by exogenous infection with virus released from neighboring, virus-positive cells. By blotting analyses of single-cell subclones, the provirus might appear to be a transposable genetic element, not mapping with any particular human chromosome,

but stably integrated only in cells containing human chromosome 6. Preliminary findings indeed indicate that the latter mechanism is most likely.

To test this possibility, we plan to counterselect chromosome 6+/virus+ hybrid clones in 5-bromodeoxyuridine (BrdU). Using parental rodent cells deficient in thymidine kinase (TK-) but coding for BEV receptors, BrdU treatment forces segregation of human chromosome 17 containing the TK gene and enhances concommitant random loss of other human chromosomes. Following BrdU exposure, hybrid cells will be subcloned, assayed for human chromosomes, and scored for BEV proviral integrations by Southern blot analyses. If chromosome six encodes a maintainance function, some chromosome 6+/virus+ subclones should demonstrate proviral integrations not detected in parental, primary hybrid colonies. In addition, rare chromosome 6+/virus- subclones might be obtained. Analogous counterselection experiments using chromosome 6+/19+/virus+ and chromosome 6+/19-/virus+ human x hamster hybrids could be used to resolve whether the mechanism of proviral rearrangement involves cell-to-cell spread of virus.

If the Bevi gene is found to be required for the maintainance of the integrated provirus, it's product presumably recognizes sequences in proviral DNA. One possibility is that the Bevi gene product will also interact with unintegreated proviral DNA intermediates. In vivo synthesis of the latter will be studied following exogenous infection of chromosome 6+/chromosome 19+ and chromosome 6-/chromosome 19+ cells, harvesting the extrachromosomal DNA at various times after infection, and studying the viral DNA content by blot analyses. For these purposes, baboon viral DNA has been cloned in bacteriophage lambda and "probes" representing different portions of the BEV genome will be employed to study the putative defects in viral DNA intermediates.

BEV cDNA probes have been seen to hybridize to background bands in human DNA. The human DNA restriction fragments reacting with the probes could represent the putative natural "targets" of the Bevi gene product. These results can be best verified using cloned proviral DNA. Methods analogous to those employed for studying BEV proviral segregation can be used to determine whether Bevi recognizes related sequences in the human genome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CP-05155-01-LVC

PERIOD COVERED
September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)

Role and Control of Recombinant Viruses in Mammalian Leukemia Model Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A series of novel murine recombinant viruses was isolated from natural and laboratory induced leukemias of mice. Each was genomically masked and biologically and biochemically unique.

Natural defense mechanisms found in mouse sera were purified as lipid in nature. The spectrum of virus inactivation potential increased with purity of this oncornavirus inactivation factor (OIF).

Seroimmunoprophylaxis experiments with anti MuLV gp70 IgG showed good protection of AKR mice if treatment occurred during a defined early period. Role of Eco and RM viruses was defined during the treatment period.

FHS-6040
(Rev. 12-79)

Project Description

Objectives:

To determine the role of recombinant MuLV's in murine leukemia. To understand the complex viral structure and the numerous permutations and changes specifically in the env region. The presence of these viruses has not been recognized until recently because of the complex mode of interactions of recombinant MuLV's, ecotropic MuLV's and natural protective factors. To examine the natural history of these viruses in disease of low and high leukemia incidence mice. To postulate a hypothesis for RM related envelope products in virus positive and negative murine leukemia. To continue examining the role of viruses in successful seriommumnoprophylaxis of natural mouse leukemia.

Methods Employed:

The usual method of tissue culture including propagation and derivation of normal and tumor cultures. Standard virological assays such as focus assay for both transforming and nontransforming oncornaviruses, soft agar assays, isotopic precursor incorporation, isolation and purification of viruses, density gradient centrifugation. Isolation of protein and nucleic acid components of viruses and cells including reverse transcriptase assays, nucleic acid hybridization, restriction enzyme analyses, and cloning of DNA fragments.

Immunological and biochemical procedures in isolation and induction of immunoglobulins and the purification of various natural host protective factors. Protein and lipoprotein analyses, radioimmune precipitation, immunofluroescent analysis of in vivo and cultured cell samples. Seroimmunoprophylaxis protocols in several in vivo model systems.

Major Findings:

Several prototype murine recombinant viruses were examined regarding the composition and products of individual virus genes. The viral envelope gene product (gp70) was identified as being different from known ecotropic and xenotropic viruses by a number of criteria, such as neutralization, interference and oligopeptide mapping. Some of these recombinant viruses were found to have either a part or the whole envelope gene exchanged or recombined. A series of new polytropic recombinant viruses were isolated from various Moloney leukemia virus stocks as well as from Gross and Graffi leukemia viruses and interestingly enough from C57 Black mouse cells uninfected with exogenous leukemia viruses. Each subset had unique properties and some of these RM's did not have classic xenotropic peptide spots. Additionally, several of these evidently have further sites of recombination in their gag

regions. This family of RM viruses was not homogenous in respect to host range properties. Some isolates were completely polytropic and some were almost ecotropic. Some could enter but not replicate in either mouse or heterologous species cells. Some were quite cytopathic for cells other than mink and some were not cytopathic at all. All known RM viruses were found to share a unique and peculiar interference subgroup. They did not cross interfere with ecotropic MuLV's but one-way interference was found with xenotropic MuLV's in that the latter could penetrate into cells preinfected with RM virus whereas RM virus could not penetrate xenotropic MuVL infected cells. Some antisera could define RM viruses as possessing subgroup specific determinants; these sera neutralized all RM viruses but not ecotropic or xenotropic MuLV's.

A salient feature of essentially all of these isolates was that they were genomically masked in nature. Basically, the recombinant virus genome was found coated with ecotropic envelopes and therefore insusceptible to type specific antibody and natural protective factors but it could, with its own envelope overcome the ecotropic virus interference barriers. Because some apparently pure ecotropic virus stocks have large amounts (>10 infectious units) of masked RM virus, purification of antigens and interpretation of immune responses must be evaluated with caution. It was clear that masking protected the RM virus from oncornavirus inactivating factor (OIF) found in normal mouse sera. This phenomenon of masking has now been observed in every MuLV leukemogenic stock tested despite a variability in growth avidity of the RM component.

Natural defense mechanisms in the mouse which are directed against xenotropic and recombinant viruses have been examined. It could be seen that potent, non-immunoglobulin mediated inactivation of xenotropic and RM viruses could occur on treatment with apparently only pure lipids found in the very light density lipoprotein fractions of rmouse sera. All mouse sera, but not other mammalian sera, had OIF, including those which were previously reported to have been negative. In the latter, OIF could be shown to be complexed into an inactive form in serum. Once purifed, OIF had a broader spectrum of activity which included some ecotropic viruses as well as some leukemia viruses of other species. No reduction of OIF could be demonstrated in pre-leukemic states in irradiation induced lymphomas of mice, or naturally arising AKR mouse lymphomas.

A recent major finding has been in the area of virus negative murine leukemia. Mice of various strains, including virus negative NIH Swiss, could be irradiated which results in a high incidence of thymic leukemias which appear not to express virus or viral antigens. We established in cultures a line of NIH Swiss mouse X-irradiation induced thymoma (NIXT) cells which were free of virus or known viral proteins. Because of this cloned cell line's insusceptibility to entry and repli-

cation of all RM viruses, but not to ecotropic viruses, it was found that these cells contained on their surface an easily removable glycoprotein (gp70) which was related primarily to RM gp's by immunological and peptide mapping techniques. This gp70 found in all subclones of NIXT cells was prominently present on 5-25% of cells, but could leach off into the supernatant. We postulated that the presence of this gp70 together with a receptor could serve as a constant auto-stimulatory blastogenesis signal which causes the uncontrolled lymphoblastic proliferation in the absence of leukemogenic MuLV's.

Goat IgG, highly reactive with type, group and interspecies specific determinants of Friend MuLV gp70 was used to treat AKR mothers and baby mice. If this was performed during the critical first nine days after birth ("window") the long term survival of the treated group was twice that of the control AKR group, i.e. two years vs. one year. Later treatment times or treatment of only mothers or baby mice was much less effective. The average long term survivors had much less virus or AKR p12 antigen, and mounted an active, type specific neutralizing and precipitating immune response against the AKR gp70. Both control and treated animals had high titers of OIF which has to be separated before immunoglobulin responses can be measured specifically against the RM-MuLV subgroup. The titer of detectable RM virus, all of which was masked by the AKR MuLV coat, and readily detectable in the control group, was very low in the surviving group and neither of the two RM isolates grew independently in heterologous cells. In a few mice, the ideal situation was as follows: no virus of any kind, no antigens, and very high precipitating and AKR neutralizing titers. These mice did not die of malignant disease. Recently breeding of long term survivors has resulted in offspring which is being tested and which appears to promise further long term survival.

Significance to Biomedical Research and the Program of the Institute:

Of particular value may be the conceptual transition from known and well defined viral mouse leukemogenesis to nonviral yet effective physical induction of leukemia with the link of a viral or virus like surface molecule. The autostimulatory, cycling blastogenesis which appears to work in AKR viral lymphoid leukemia may be of real significance if this concept also holds true in other nonviral leukemia models. Specific attention could then be focused on methods employing NIXT gp70 specific antibody (monoclonal?) to see whether nonviral disease modification could result.

If preparations of OIF can be made broadly reactive in a relatively potent fashion, then these could be used in various treatment regimens especially directed against a series of lipid containing viruses.

Despite the apparent simplicity of the seroimmunoprophylaxis system, it represents a functional, holistic and successful approach to the

treatment of an overwhelming viral disease. As envisioned the model system will be defined in all its components, and specific additional inputs, both protective and detrimental, could be evaluated on a well worked out background. These may involve further cellular immunotherapy as well as accepted additional chemotherapeutic regimens.

Proposed Course:

A correlation of the defined recombination site in the env region as measured by oligopeptide or oligonucleotide patterns will be made to identify the sequence(s) or determinant(s) responsible in the shift of host range, interference, and RM subgroup specific neutralization. The role of specific sites associated with leukemogenesis could be possible if interpolated relative to the common conserved regions. The natural history of the expression of these viruses will be studied in spontaneous AKR mouse leukemogenesis and in further examples of non-viral physical leukemogenesis in NIH Swiss mice. The isolated gp70 from NIXT cells will be used to generate specific antisera, and the message for this protein will be examined with RNA blotting technology. Additional non virus associated animal model systems will be examined with the possibility that lymphoid leukemogenesis may be brought about by analogous antigen receptor systems. The specific origin, e.g. bone marrow, thymus, and the timing of the recombinant event, appear to be important as well as the frequency and non-randomness of such events. So far, reconstruction experiments in tissue culture have never been successful (in more than 2 years) in generating a typical RM virus. The physiochemical nature of OIF will be further investigated in collaborative studies to determine the mechanism of action and binding properties to various MuLV's and their antigenic determinants. Because of the initial findings of an extended spectrum of virus inactivation, modification of OIF structure will be attempted to amplify this phenomenon.

Publications:

Montelaro, R. C., Fischinger, P. J, Larrick, S. B., Dunlop, N. M., Ihle, J. N., Frank, H., Schafer, W., and Bolognesi, D. P.: Further characterization of the oncornavirus inactivating factor in normal mouse serum. Virology 98: 20-34, 1979

Fischinger, P.J., Ihle, J. N., Levy, J. P., Bolognesi, D. P., Elder, J., and Schafer, W.: Recombinant murine leukemia viruses and protective factors in leukemogenesis. In: Viruses in Naturally Occurring Cancer. Cold Spring Harbor Symp. Quant. Biol., in Press, 1979.

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